B-VITAMINS

FOR BLOOD FORMATION

THOMAS H. JUKES, Ph.D.

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1. megaloblastic anemias
2. B vitamins ③ pregnancy
4. infancy ⑤ folic acids
6. pteroylglutamic acids
7. vitamin ⑩ B12
8. Citrovorum factor
9. intrinsic "
10. anemia ⑫ metabolic reactions
11. Vitamin B6 deficiency

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For Blood Formation

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PREFACE

Biochemical progress in the past six years has led to a surge of interest in the megaloblastic anemias and new problems have been posed which are the subject of many discussions in the scientific literature. It is now evident that the processes involved in the production of blood cells have certain chemical relationships to general proliferative changes involving the formation of nuclear material.

In 1945, a new B-complex vitamin, pteroylglutamic acid, was synthesized. This substance was found to be needed for the prevention of deficiency states which had been described as occurring in a variety of animals and micro-organisms. Pteroylglutamic acid was shown to be needed for the cure of certain anemias of dietary origin in human subjects. Its abundant availability in the synthetic form led to a new era in the treatment of sprue and the megaloblastic anemias of infancy, pregnancy and pellagra. Pteroylglutamic acid is a stable, yellow, crystalline substance which is well utilized when given either by mouth or by injection in doses of a few milligrams daily. It is widely distributed in foods of both animal and vegetable origin such as liver, yeast, green leaves and soybeans.

The essential role of pteroylglutamic acid in the formation of blood cells has its counterpart in the activity of various synthetic "antagonists" of this vitamin in depressing cytopoiesis. These new substances were also found to produce an accentuated form of the other manifestations of pteroylglutamic-acid deficiency. The antagonists have been

extensively studied in attempts to manage leukemia and other neoplastic diseases.

Pernicious anemia was first treated successfully with whole liver in 1926, but today there is no certainty as to which of the now-known hemopoietic factors in liver was responsible for the original remissions. Shortly afterwards injectable liver extracts were prepared for the treatment of this disease and it became evident that these extracts had very little effectiveness when given by mouth in contrast to their potency when injected. An apparently unique biological mechanism then came to light; normal gastric secretions were shown to contain an "intrinsic factor" which is needed for the uptake of a dietary "extrinsic factor" from the digestive tract. The extrinsic factor has been identified with a group of substances, the "cobalamins," which are present in many foods of animal origin and are formed by numerous micro-organisms. The mechanism by which the intrinsic factor facilitates the passage of the cobalamins through the intestinal wall is still unknown. The human being who is afflicted with pernicious anemia, a disease peculiar to his species, is deprived of the intrinsic factor by a degenerative process which arrests the formation of normal gastric juice, thus he most literally starves in the midst of plenty. Indeed, injectable and therapeutically active solutions of cobalamins may be prepared from the stools of such patients, whose excreta may actually contain more potency than those of normal subjects.

The isolation of crystalline vitamin B_{12} was described in 1948. This substance is also termed "cyano-cobalamin" and is representative of the cobalamins, which consist of a large and intricate cobalt-containing molecule coordinated with various anions such as cyanide and hydroxyl. The cobalamins are effective in producing remissions in pernicious anemia when injected in quantities as low as 1 to 2

micrograms daily. The use of the crystalline substances in nutritional experiments confirmed earlier evidence that the anti-pernicious-anemia factor of concentrated liver extracts was identical with a vitamin needed by animals. This vitamin was known to be present in such foods of animal origin as fish, lean meat, milk and liver. These discoveries were overshadowed in practical importance by the finding in 1948 that the cobalamins were formed by many bacteria and especially by the *Streptomyces* group of microorganisms. Industrial fermentations were able to provide large quantities of cobalamins for use in animal feeding and in pharmaceutical products.

Studies with lactic acid bacteria led to the recognition of a hitherto unknown "citrovorum factor" which was shown to be a modified form of pteroylglutamic acid with certain biological activities exceeding those of the latter substance. Chemical experiments resulted in the discovery of the structural characteristics of the "citrovorum factor" which evidently is widely distributed in biological materials and which appears to be concerned in the biochemical transfer of "single-carbon" fragments. The factor has been synthesized and has anti-anemic properties resembling those of pteroylglutamic acid.

This short monograph is devoted to a field of study which continues to expand so rapidly that reviews of the subject are soon out-of-date. I am indebted to Miss Josephine Block for her help in the preparation of the manuscript. Grateful acknowledgment is also made to Dr. Byron E. Hall, Dr. Robert P. Parker and to Mr. C. Maresh for furnishing the material which is used in the illustrations.

T. H. J.

Pearl River, New York
June, 1951

CONTENTS

B - VITAMINS

For Blood Formation

CHAPTER I

INTRODUCTION —THE MEGALOBLASTIC ANEMIAS

THE PRESENCE of anti-anemic substances in mammalian liver has long been recognized but the purification and isolation of the active principles eluded laboratory investigators for many years. The use of microbiological assay procedures was of great importance in the isolation of these substances, which belong to the group of B-complex vitamins, naturally-occurring biochemical catalysts or coenzymes. During the past few years rapid developments have taken place in knowledge of the chemistry of certain substances which are concerned with the formation of blood cells.

Pteroylglutamic acid was synthesized in 1945 and this compound was widely investigated with respect to its effects in producing a response in anemias which are accompanied by megaloblastic arrest in the bone marrow. Two years later chemical analogs of pteroylglutamic acid were synthesized which are biologically antagonistic to their prototype so that they produce an acute deficiency disease, often accompanied by anemia and leukopenia, in experimental animals.

Vitamin B_{12}, another substance effective against certain megaloblastic anemias, was isolated in 1948. Chemically vitamin B_{12} and pteroylglutamic acid are quite different

but they are closely interrelated in certain biochemical processes. Both substances are involved in the formation of desoxyribonucleic acid as shown by their relation to the biological synthesis of purine and pyrimidine bases and desoxyribosides.

Various schemes have been proposed for classification of the anemias based either on etiology or on cell morphology. The complexity of the situation may be gauged by reference to textbooks of hematology [1] which summarize the years of effort and study which have been devoted to an understanding of the causes and pathology of disorders of the blood and blood-forming organs. The experimental use of drugs continues to throw light on the biochemical nature of the causes of blood dyscrasias. It is the purpose of this monograph to review the chemistry and physiology of some of the newer therapeutic agents in this field. Consideration is given to (a) Pteroylglutamic acid and its chemical relatives; (b) The vitamin B_{12} group; and (c) The "intrinsic factor."

THE MEGALOBLASTIC ANEMIAS

Interest among nutritionists has centered in a group of anemias characterized by megaloblastic erythropoiesis, a similar disturbance in the myeloid series with pathologic macro-myeloid cells and a reduction in number and abnormality in type of thrombocytes.[2] This group comprises the megaloblastic anemias and includes pernicious anemia, sprue and certain other anemias, one of which has been termed "tropical macrocytic anemia." [2, 3] Wills used this name to describe a megaloblastic anemia occurring during pregnancy and not responding to treatment with purified liver extracts known to be effective in pernicious anemia. Tropical macrocytic anemia was alleviated by

the administration of an extract of autolyzed yeast, "Marmite." The existence of two dietary factors which were effective against the megaloblastic anemias was demonstrated by these findings. The first of these, present in concentrated liver extract, was eventually shown to be vitamin B_{12}. The second factor, present in liver and yeast, was found to be folic acid.

Pernicious anemia is accompanied by a failure in the function of gastric mucosa so that the tissue no longer secretes hydrochloric acid even when stimulated by the injection of histamine. The gastric juice in pernicious anemia is further characterized by lacking a specific protein-like substance, the "intrinsic factor," which is present in normal gastric juice and which is evidently needed for the normal uptake of vitamin B_{12} from the digestive tract. A deficiency of vitamin B_{12} is consequently developed by the tissues in this disease and is marked by a macrocytic anemia, specific changes in the bone marrow, and usually by subacute combined degeneration of the spinal cord. Among the physical signs are "lemon yellow" pallor; a clean tongue, either very red with a smooth, swollen shiny tip and lateral portions, or pale, shrunken, smooth and shiny all over; impaired sense of position and vibration and a positive Romberg sign, and retinal pallor with flame-shaped retinal hemorrhages. Important laboratory findings are macrocytic anemia with a mean corpuscular volume greater than 97 cubic microns and color index usually greater than 1; gastric achlorhydria with no free acid in four samples of gastric juice aspirated at intervals of 15 minutes after injection of 0.5 mg. of histamine; increase of nucleated red cells, including more than 2 per cent megaloblasts, in films prepared from fluid aspirated from sternal bone marrow; leucopenia with relative lymphocytosis, hypersegmentation of nuclei of neutrophils and moderate decrease in platelets and increase in

concentration of serum bilirubin which gives an indirect Van den Bergh reaction.[4] The signs and symptoms are relieved by oral administration of a mixture of vitamin B_{12} with the "intrinsic factor." Relief of the cord symptoms appears to be dependent upon the degeneration of the nervous tissue not having progressed to an irreversible state.

Before the isolation of pteroylglutamic acid, which in turn preceded the isolation of vitamin B_{12}, it was evident that folic acid deficiency in animals was not alleviated by concentrated liver extracts, indeed the manufacturing processes employed in the production of such extracts led to the discarding of the folic acid content of crude liver extracts in "side fractions." The absence of significant quantities of folic acid activity from concentrated liver extracts indicated that it was not to be anticipated that pteroylglutamic acid would be effective in pernicious anemia. However, this substance produced a typical hemopoietic response in this disease whether administered parenterally or orally and in the latter case regardless of the absence of the "intrinsic factor." The central nervous symptoms did not respond to pteroylglutamic acid. Pernicious anemia may develop even though "normal" diets are consumed, so these findings led to speculation and experimentation upon the nutritional availability in pernicious anemia of the conjugated forms of pteroylglutamic acid which are present in foods, but definite conclusions cannot be said to have been reached. An interplay between pteroylglutamic acid and vitamin B_{12} has been observed in certain metabolic processes, and it may well be that the riddle of the interchangeable hemopoietic roles of pteroylglutamic acid and vitamin B_{12} in pernicious anemia will be solved only through studies of biochemical reactions.

The therapeutic effect of pteroylglutamic acid upon

the megaloblastic anemias of infancy, pregnancy and pellagra appears to be clear-cut. These anemias are typically associated with a dietary deficiency of folic acid which in the cases of pregnancy and infancy may be aggravated by the high requirement for vitamins during rapid growth. Achylia gastrica is not present so that the uptake of vitamin B_{12} from the digestive tract is not specifically impaired in these anemias as appears to be the case in pernicious anemia. The existence of megaloblastic anemias which do not respond to concentrated liver extracts has drawn the attention of various investigators and the names "refractory megaloblastic anemia" [5, 6] and "achrestic anemia" [6, 7] have been applied to conditions of this type. Davidson [5] has found that "refractory megaloblastic anemia" responds well to folic acid.

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CHAPTER II

B VITAMINS IN THE MEGALOBLASTIC ANEMIAS OF PREGNANCY AND INFANCY

PTEROYLGLUTAMIC ACID AND VITAMIN B_{12} IN THE MEGALOBLASTIC ANEMIA OF PREGNANCY

THE MEGALOBLASTIC anemia of pregnancy is characterized by the presence of macrocytic red blood cells, by megaloblastic changes in the bone marrow,[1] by the presence of hydrochloric acid in the gastric juice and by the absence of neurological signs and symptoms. The disease may occur at any age during the childbearing period in both primigravidas and multigravidas. Most patients have a defective diet. Symptoms usually arise during the third trimester or in the puerperium but occasionally they date from early pregnancy or miscarriage. Excessive vomiting or diarrhea is noted in about half of the cases. The tongue may be sore and slight edema is frequent. A "pearly white" appearance is common and a yellowish pallor is rare. The blood picture is variable; leucopenia is common, and the marrow usually shows a mixed megaloblastic and normoblastic reaction.[1]

The anemia tends to disappear spontaneously after the termination of pregnancy. The early observations by Wills which served to differentiate the curative agent from the anti-pernicious-anemia factor of concentrated liver extract

were reviewed on page 4. Similar findings prior to the availability of pteroylglutamic acid were reported by various clinical groups [2-19] who noted the curative effects of various crude fractions, especially those prepared from liver and yeast.

In 1945 Moore and co-workers [20] described the use of pteroylglutamic acid in the treatment of a patient with the megaloblastic anemia of pregnancy. She had a red blood cell count of 1.1 to 1.2 million per cu. mm. 18 days after parturition. A daily intramuscular dose of 20 mg. was given for 10 days, and a peak value of 48 per cent reticulocytes was reached on the seventh day. The red cell count rose rapidly and the clinical response seems to have been complete. The successful treatment of three patients with 20 to 50 mg. of pteroylglutamic acid daily was described by Spies.[21] The patients showed reticulocyte peaks of 23 to 29 per cent. Three cases which had failed to respond to the injection of concentrated liver extracts were treated with pteroylglutamic acid by Davidson and co-workers and all responded promptly.[22] Analogous results were described in India by Benjamin-Allan.[23]

Shortly after the isolation of vitamin B_{12}, it was reported by Ungley that a case of the megaloblastic anemia of pregnancy failed to respond to 65 micrograms of vitamin B_{12} but the patient subsequently responded to 2.5 mg. of pteroylglutamic acid daily.[24] A failure with vitamin B_{12} was described by Bethell and co-workers,[25] who noted that anemia and leucopenia became more severe and mucous membrane lesions progressed during treatment with vitamin B_{12} but later disappeared when pteroylglutamic acid, 10 mg. daily, was given. A striking case was reported by Day *et al.*[26] The patient had loss of hair, swollen feet, legs and hands and a waxy, pearly-white color of the skin. The stools were watery and numerous, while "the mucous

membranes of the gums and cheeks were brick red and a striking contrast to the pale tongue." The hemoglobin was 6.5 per cent, the red cell count was 1.7 million, and other diagnostic signs of the megaloblastic anemia of pregnancy were recorded. Liver extract was given for 15 days, followed by eight days of vitamin B_{12} treatment which appeared to exacerbate the sore gums and the diarrhea. Administration of pteroylglutamic acid was then initiated and within 24 hours she became exceedingly hungry and

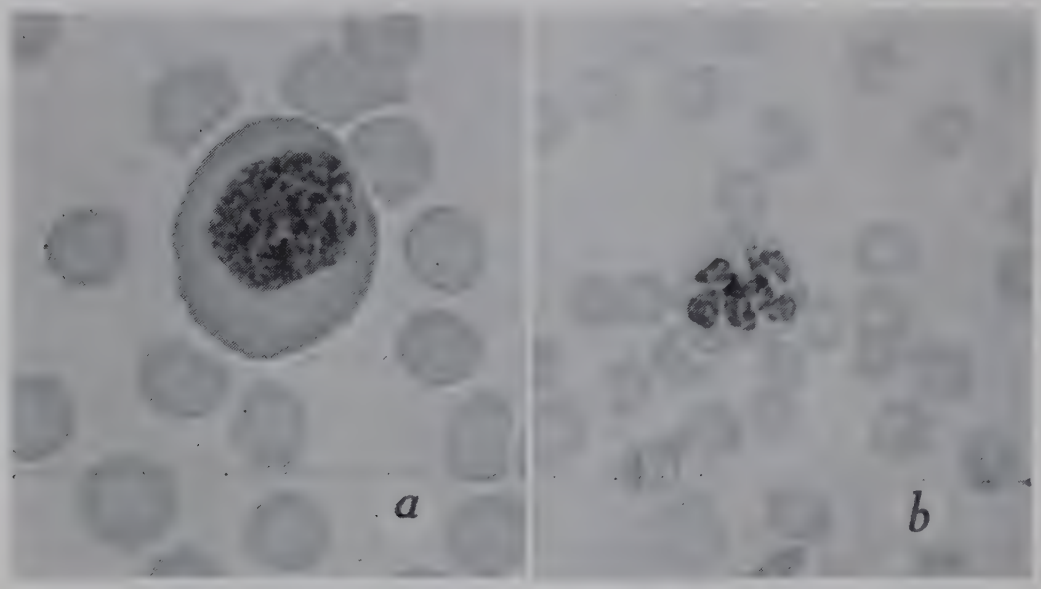

Figure 2.1. Megaloblast (x1000) in peripheral blood before treatment with folic acid. (From Day and co-workers.[26])
Figure 2.2. Hypersegmented polymorphonuclear leucocytes (x700) from same blood as Figure 2.1. (From Day and co-workers.[26])

ingested large amounts of food. The edema receded and the diarrhea disappeared; the hair stopped falling out and the texture of the skin changed from dry and scaly to smooth and soft. The patient felt entirely well after two weeks of treatment and a concomitant return to normal was found in the hemopoietic system. The case is illustrated in Figures 2.1, 2.2, 2.3, and 2.4. The report is noteworthy for its detailed description of acute and uncomplicated pteroylglutamic acid deficiency in a human subject.

The susceptibility to pteroylglutamic acid deficiency which is increased in women during pregnancy finds its counterpart in "lactation leucopenia" in rats.[27]

MEGALOBLASTIC ANEMIA OF INFANCY

A megaloblastic anemia in infants was noted by Faber [28] who described typical abnormalities in the bone marrow occurring as early as six weeks of age. "Goats' milk anemia" in infants was often described in the German literature and

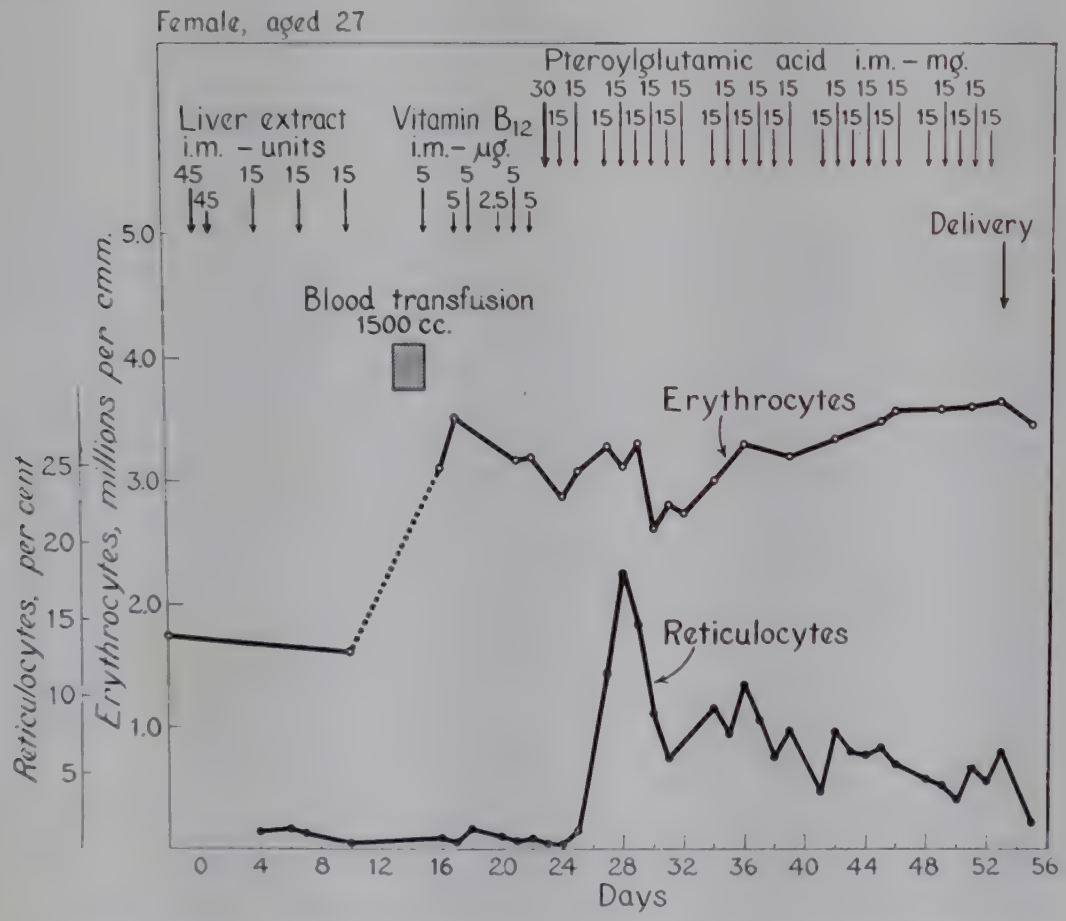

Figure 2.3. Absence of hematopoietic response to liver extract and to vitamin B_{12} and a prompt response to pteroylglutamic acid. The total dose of liver extract administered for 15 days amounted to 135 units; the total dose of vitamin B_{12} given over a period of eight days was 27.5 micrograms, and the total dose of pteroylglutamic acid given over a period of 29 days was 405 milligrams. (From Day and co-workers.[26])

studies by Gyorgy [29] and by Rominger and co-workers [30] indicated that a B-complex factor in liver and yeast would cure the condition.

A report by Zuelzer and Ogden in February, 1946 [31] contained a description of the megaloblastic anemia of infancy and the first account of the curative action of pteroylglutamic acid for this condition. The disease [32, 33] was characterized by a normochromic and usually macrocytic anemia, a tendency toward leucopenia and neutropenia, a diminution of platelets, often associated with an increased bleeding tendency, a megaloblastic bone marrow pattern, a frequent incidence of splenomegaly, and a histamine-refractory achlorhydria present but reversible following remission. The treatment of 12 patients with pteroylglutamic

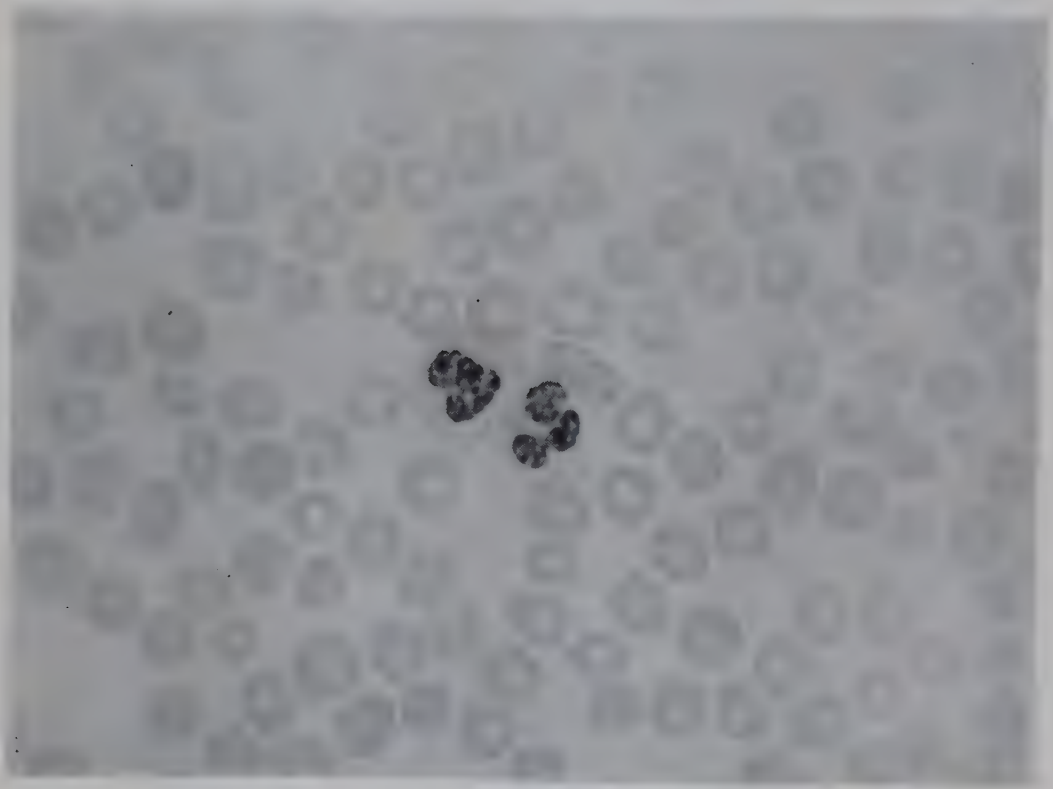

Figure 2.4. Peripheral blood seven days after the beginning of treatment with pteroylglutamic acid showing uniform size and shape of red cells and normal polymorphonuclear cells (x700). (From Day and co-workers.[26])

acid either crystalline or in the form of a natural concentrate prepared by eluting a charcoal adsorbate of a liver fraction was described. The dosage rate was 5 to 20 mg. daily for periods ranging from eight to 21 days. Three patients died with severe infections but the others responded, showing reticulocyte peaks, a return of the bone marrow pattern to normal, and increases in hemoglobin, red cell counts, and platelets counts. A secondary reticulocyte peak was not obtained when ascorbic acid was administered following treatment with pteroylglutamic acid. No relapses were observed in follow-up studies which lasted up to 10 months. In a subsequent discussion, Zuelzer [34] pointed out that 21 out of 36 cases had received formulae made from diluted dried cows' milk. Two others had been fed on goats' milk and another two on breast milk. The mothers of the breast-fed infants were both anemic. Of much interest was the observation that eight of the 36 cases had scurvy. Zuelzer stated that either liver extracts or folic acid were equally satisfactory in effecting complete and lasting remissions. A series of 25 cases were described in Italy by Amato [35] in breast-fed infants without supplementary feedings.

It was stated by Siebenthal [36] that "megaloblastic anemia is a relatively common disease of white infants and since a complete and permanent recovery can be effected by folic acid therapy, the condition should be understood by all those caring for small children."

Observations on the development of megaloblastic anemia in infants receiving dried milk formulae were reported by Luhby and Wheeler.[37] Crystalline vitamin B_{12} was without effect in three cases but excellent responses were obtained to pteroylglutamic acid and in one case to spinach, which is a good source of pteroylglutamic acid. In another case, ground beef incubated with normal gastric

juice was ineffective and the patient subsequently responded to pteroylglutamic acid in a single intramuscular dose of 0.8 mg. with a reticulocyte peak of 32 per cent. Somewhat in contrast, McPherson and co-workers [38] found a response to vitamin B_{12} in two infants but recovery was slower than with pteroylglutamic acid. Woodruff and co-workers [39] studied five infants, three of whom responded to vitamin B_{12} but the other two showed no improvement and responded only after pteroylglutamic acid had been administered. Sturges and Carpenter [40] noted a good response to vitamin B_{12} in three cases but in two other infants the responses were not clear-cut.

The relation of ascorbic acid deficiency to the megaloblastic anemia of infancy was discussed by May and co-workers [41] and by Zuelzer and co-workers.[42] May reported that two infants with megaloblastic anemia had a history of ascorbic acid deficiency. Administration of ascorbic acid did not correct the megaloblastosis, but the patients promptly responded to liver extract. Zuelzer observed that good hemopoietic responses were obtained with pteroylglutamic acid in the presence of continued ascorbic acid deficiency but no secondary reticulocyte response followed the subsequent administration of ascorbic acid. He concluded that ascorbic acid deficiency *per se* did not lead to anemia. Further discussion was contributed in 1950 by May and co-workers.[43] They drew attention to the following points:

1. Megaloblastic anemia has been reported frequently as a complication of scurvy.

2. Patients with pernicious anemia have been encountered who were said to be refractory to liver extract until ascorbic acid was administered.

3. Either pteroylglutamic acid or ascorbic acid will

relieve the tyrosyluria induced in scorbutic guinea pigs by feeding excessive tyrosine.

4. Megaloblastic anemia had not been produced by feeding a wide variety of deficient diets to species which are able to synthesize ascorbic acid.

5. Wills and Day had produced macrocytic anemia in monkeys with diets devoid of animal protein or containing vitamin-free casein, but the animals were protected from anemia if milk or ordinary casein and ascorbic acid were included in the diets.

Some objections may be raised to some of these arguments. Pteroylglutamic acid has been described as not relieving tyrosine-induced hydroxyphenyluria in scorbutic infants.[44] Macrocytic anemia has been produced in chicks and turkeys by feeding diets deficient in pteroylglutamic acid.[45] Milk and "ordinary" casein are recognized sources of vitamin B_{12}. Ascorbic acid was present in the basal diet used for monkeys by Wilson and co-workers,[46] who reported that macrocytic anemia developed and was relieved by pteroylglutamic acid. However, May and co-workers concluded from their studies with monkeys that when ascorbic acid was provided adequately, the diets did not lead to megaloblastic anemia which occurred when the supply of ascorbic acid was inadequate. Pteroylglutamic acid but not vitamin B_{12} prevented the development of megaloblastic bone marrow in the monkeys. The central role of pteroylglutamic acid in the treatment of the megaloblastic anemia of infancy was emphasized. These observations take on added interest in view of the finding by Welch and Nichol regarding the augmentation produced by ascorbic acid in the formation of citrovorum factor in liver slices in the presence of pteroylglutamic acid (Chapter VII).

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CHAPTER III

FOLIC ACID : PTEROYLGLUTAMIC ACID

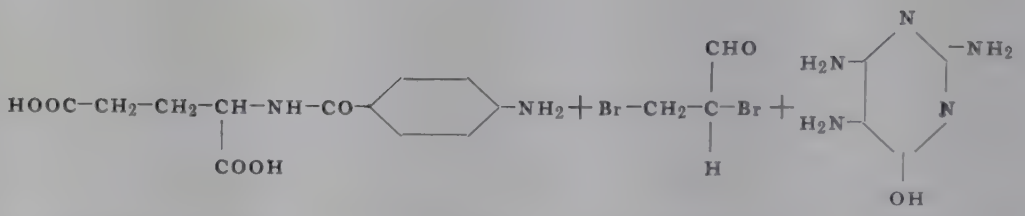
The name "yeast norite eluate factor" was applied by Snell and Peterson [1] to a vitamin-like factor which was needed by *Lactobacillus casei* for growth on a purified culture medium. These studies were continued by Mitchell, Snell and Williams [2] who re-named the factor "folic acid" and defined in it terms of activity for *Streptococcus faecalis* R. Further investigations [3, 4, 5] led to the isolation, degradation and synthesis of the substance, which was named "pteroylglutamic acid" in view of its chemical structure (Figure 3.1). The names "folic acid" and "pteroylglutamic acid" are often used interchangeably, but biological activity for *S. faecalis* R is shown by a number of substances in addition to pteroylglutamic acid including pteroic acid, N^{10}-formylpteroic acid, N^{10}-formylpteroylglutamic acid, pteroyltriglutamic acid and the "citrovorum factor group." Any one of these substances will give a response with *S. faecalis* R under the conditions originally laid down for the test, and hence the substances have "folic acid activity" (Table 3.1). The term "pteroylglutamic acid" (PGA) is used in this monograph to refer specifically to the substance and "folic acid activity" to refer to the biological activity of natural materials which may or may not be due to pteroylglutamic acid. Table 3.1 also shows the activity of the various substances for *L. casei*, and indicates that the pattern of response of this organism differs from that of *S. faecalis*.

TABLE 3.1

Activity of Various Substances Related to Pteroylglutamic Acid for *S. faecalis* and *L. casei* in Terms of Pteroylglutamic Acid = 100

Substance	*Activity for S. faecalis and R L. casei*	
Pteroic acid	50 to 100	0.01
Pteroyl-γ-glutamylglutamic acid	100	100
Pteroyltriglutamic acid (Pteroyl-γ-glutamyl-γ-glutamylglutamic acid)	7.5	80
N^{10}-formylpteroic acid	125	very low
N^{10}-formylpteroylglutamic acid	50	50
Leucovorin	50	50
Pteroylheptaglutamic acid (Vitamin B_c conjugate)	0.3	0.2

Pteroylglutamic acid may be synthesized by condensing 2,4,5-triamino-6-hydroxypyrimidine with 2,3-dibrompropionaldehyde and *p*-aminobenzoyl(L)glutamic acid [5] (Figure 3.1).



p. aminobenzoyl(L)glutamic acid dibrompropionaldehyde 2, 4, 5-triamino-6-hydroxypyrimidine

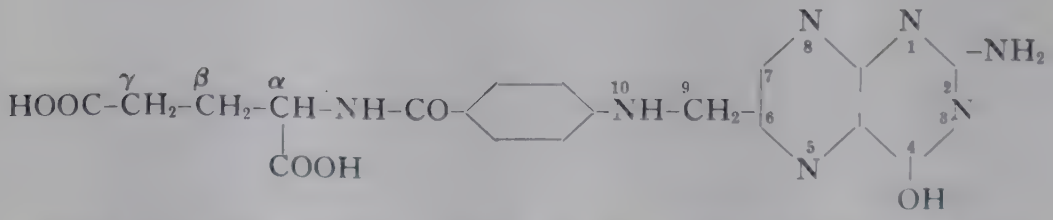


Figure 3.1. Pteroylglutamic acid; (N-(p-((2-amino-4-hydroxy-6-pteridylmethyl)amino)-benzoyl)glutamic acid), and its synthesis.

Figure 3.2. Pteroylglutamic acid crystals; photomicrograph through crossed polarizers, x800. (Stamford Research Labs., American Cyanamid Co.)

PROPERTIES

Pteroylglutamic acid is a yellow compound which crystallizes from water as yellow spear-shaped leaflets (Figure 3.2). On heating it does not melt but chars at around 250°. The specific rotation $(\alpha)_c^{20°}$ is +16°, measured in 0.1N sodium hydroxide solution, concentration 0.76 gram per 100 ml.[6] Only 1 mg. dissolves in 100 ml. of water at 0° C. and 50 mg. at 100° C. but the disodium salt ("sodium folate") has a solubility of over 1.5 grams per 100 ml. Pteroylglutamic acid is practically insoluble in most organic solvents, but is slightly soluble in acetic acid. Drying of pteroylglutamic acid at moderate temperatures and atmospheric pressure leaves an amount of water corresponding roughly to a dihydrate, but crystallographic studies indicate that it is not a true hydrate.[7] The compound has characteristic ultraviolet absorption spectra and the curve in alkaline solution with the prominent bimodal maxima at 256 and 282 mμ is shown in Figure 3.3. Catalytic hydrogenation in dilute alkali produces dihydropteroylglutamic acid by reduction of the pyrazine ring. The dihydro form is easily reconverted to pteroylglutamic acid by atmospheric oxygen. Reduction with zinc and acid cleaves the 9,10-linkage and *p*-aminobenzoylglutamic acid is formed. Exposure of solutions of pteroylglutamic acid to daylight leads to a similar cleavage which proceeds most rapidly at pH 7.0.[8] Further studies of the photolytic reactions were made by Lowry and co-workers[9] who found that when pteroylglutamic acid was irradiated with ultraviolet light in slightly acid solution it underwent oxidative cleavage to yield successively 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxy-6-carboxypteridine and 2-amino-4-hydroxypteridine. The formylpteridine, as

also shown elsewhere [10] was an extremely active inhibitor of xanthine oxidase; this enzyme could convert the final product, 2-amino-4-hydroxypteridine, to isoxanthopterin.

PTEROYLGLUTAMIC ACID CONJUGATE

Pteroylglutamic acid appears to be present in natural materials largely in the form of a polyglutamic acid peptide containing seven glutamic acid residues in γ-linkage which has been termed "vitamin B_c conjugate," "pteroylglutamic acid conjugate," "pteroylhexaglutamylglutamic acid" [11] and "pteroylheptaglutamic acid." [12] This compound was first isolated from yeast by Pfiffner and coworkers [13] and its activity for *L. casei* and *S. faecalis* was shown to be quite low. In contrast its potency for chicks

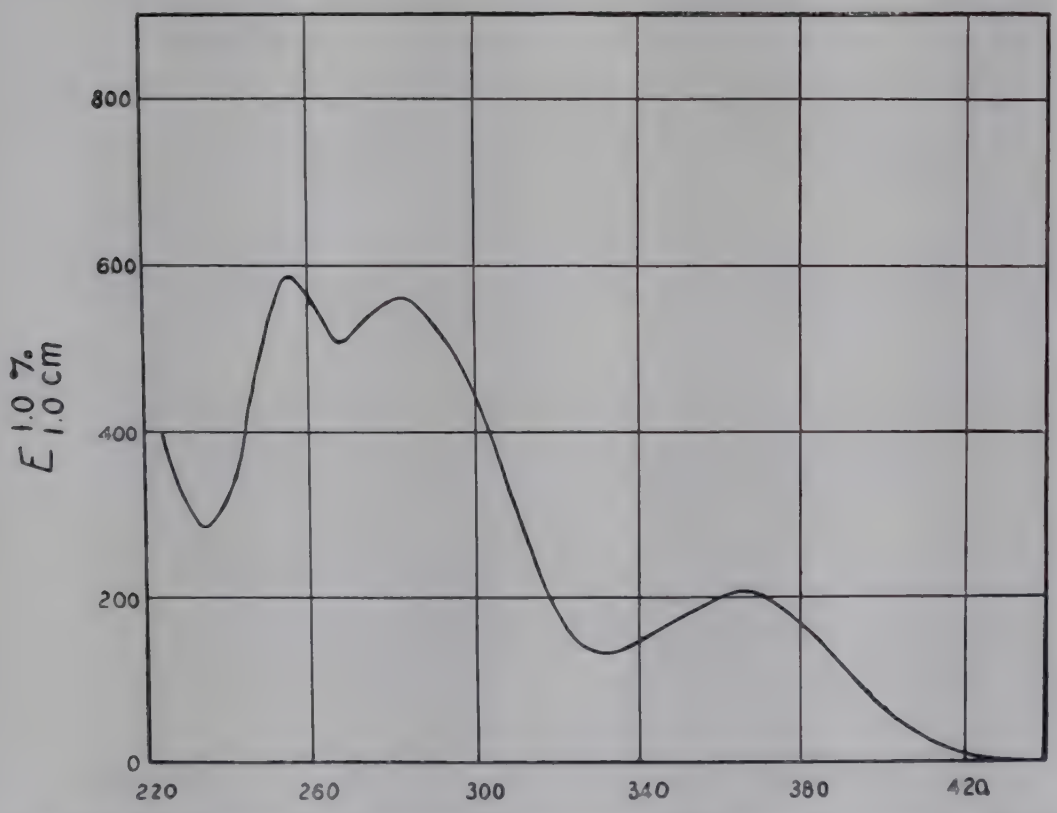


Figure 3.3. Ultraviolet absorption spectrum of pteroylglutamic acid in alkaline solution.

and rats was found to be equivalent to its content of pteroylglutamic acid. This finding served finally to resolve the disparities which existed between the microbiological and animal responses to certain natural sources of folic acid.[14] It had been found possible to prepare a dialyzable fraction from yeast which was active for deficient chicks but which had relatively little potency for *L. casei* until the fraction was subjected to an enzymatic digestion which liberated a microbiologically active substance.[15] The existence of an enzyme in rat liver which was capable of liberating folic acid activity from various natural materials was demonstrated by Mims and co-workers.[16] "Difco" yeast extract was an outstanding source of the enzymatically-liberatable activity. The enzyme system was termed "vitamin B_c conjugase" and was found present in a variety of natural sources including hog kidney, liver, small intestine, beef liver, sweet almonds,[17] various rat tissues, rabbit and chicken brain, chicken liver, intestinal mucosa and pancreas.[18] As a convenient and comparatively rich source, chicken pancreas was used for concentration of the enzyme and increase in potency of more than 1000-fold was obtained by adsorption and precipitation techniques. The optimum pH for the action of the enzyme was between 7 and 8.[18] Precipitation at between 40 and 80 per cent saturation of ammonium sulfate was used in further purification studies. The concentrated preparation was activated by calcium, and the optimum pH and temperature were found to be 7.8 and 32°.[19] In contrast, the enzyme present in hog kidney and certain other tissues had an optimum pH of 4.5 and temperature of 45° to 48°. An inhibitor was found to be present in yeast extract.

The simultaneous existence in animal and vegetable tissues of pteroylglutamic acid conjugate and the enzyme which liberated folic acid activity from it gave rise to much

biochemical interest, and various investigations of the enzyme and its substrate were described. The presence of a conjugase inhibitor in the diet did not decrease the efficiency of utilization of pteroylglutamic acid conjugate for rats as measured by leucocyte maturation,[20] but feeding the inhibitor appeared to diminish the urinary extraction of pteroylglutamic acid following the administration of the conjugate. Kazenko and Laskowski [21] reported that at least two terminal glutamic acid molecules in the peptide chain were required for the action of the enzyme which they classified as a γ-glutamic acid carboxypeptidase. The use of a preparation of the chicken pancreas enzyme was widely adopted as a means for liberating activity preliminary to the assay of the folic acid content of natural materials with *S. faecalis* R.[22]

Olson and co-workers [23] found that the folic acid activity of rat liver homogenates was increased by autolysis at pH 7 but was not increased by autolysis at pH 4.5 or by digestion with hog kidney conjugase. This indicated the existence of a precursor of folic acid other than the conjugate. Previous work had also shown that when rat liver was autolyzed at various pH values a maximum amount of folic acid activity was liberated at pH 7.[24]

EARLY NUTRITIONAL STUDIES OF FOLIC ACID DEFICIENCY

An anemia in pregnant women was described in 1931 by Wills [25] who found that the blood picture in the disease was similar to that encountered in pernicious anemia and that good responses were obtained to the oral administration of a concentrated extract of autolyzed yeast. A deficiency disease, accompanied by macrocytic anemia, could be produced in monkeys which received a diet similar to

that consumed by the human patients. The deficient monkeys responded to crude extracts of yeast or liver, but neither the animals nor the human subjects responded to purified liver extracts which were effective in the treatment of pernicious anemia.[26, 27, 28] These observations undoubtedly dealt with the development and cure of dietary pteroylglutamic acid deficiency and with its failure to respond to vitamin B_{12}.

Another series of investigations was started in 1935 with monkeys by Day and co-workers,[29] who used a simplified diet of polished rice, ground wheat, purified casein, cod liver oil, salt mixture and orange. The animals developed "nutritional cytopenia," marked by anemia, leucopenia, ulceration of the gums, diarrhea and susceptibility to bacillary dysentery and to various experimental infections. An unidentified "vitamin M," present in yeast and liver, was needed for the prevention of nutritional cytopenia.[30] A response was obtained to a "folic acid concentrate" prepared from yeast autolysate.[31] It was found that intramuscular injection of 4 or 4.5 mg. of a concentrated preparation of pteroyltriglutamic acid over a period of a few days resulted in prompt and complete remission with return of the total white cell and granulocyte counts to normal levels. The reticulocyte responses of the monkeys to pteroyltriglutamic acid were much greater than the commonly accepted values for the responses of pernicious anemia patients to adequate therapy.

In another approach, it was found by Stokstad and Manning [32] that an unidentified vitamin, "factor U," present in yeast was needed for the growth of chicks on a simplified diet. A macrocytic hyperchromic anemia was observed in chicks on a similar diet by Hogan and Parrott who termed the preventive factor "vitamin B_c." [33] The factor was eventually crystallized, using liver as a starting material [3] and was identical with pteroylglutamic acid.

A level of 0.4 parts per million of diet was sufficient for normal hemoglobin, hematocrit, red cell count and thrombocyte values in chicks, but a level of about four parts was required for normal leucocyte levels.[34] Subcutaneous injection was only slightly more effective than oral administration.[35]

Rats were also used in early studies which were shown later to be concerned with folic acid deficiency. It was found that the addition of any of several sulfonamides to a purified diet containing no source of folic acid resulted in the appearance of agranulocytosis, leucopenia, anemia, hypocellularity of the bone marrow and slow growth which were corrected by sources of pteroylglutamic acid.[36, 37] The deficiency was in all likelihood due to a reduction of the numbers of certain intestinal bacteria or to a change in their nature by the sulfonamides; these bacteria normally produce substances with folic acid activity. The use of purified diets without sulfonamides also produced blood dyscrasias due to folic acid deficiency in rats. In one report, granulocytopenia was produced in a small percentage of rats[38] and in another investigation "lactation leucopenia" was produced in rats on purified diets as a result of the additional physiological strain imposed by pregnancy and lactation.[39] Both conditions were corrected by pteroylglutamic acid.

The association of nutritional cytopenia with a lack of pteroylglutamic acid is universal in all species of animals in which the deficiency has been produced. Mice, turkeys, mink, foxes, pigs and dogs all develop anemia and leucopenia, usually accompanied by hypoplastic bone marrow changes. In some of these species the deficiency was experimentally induced by the administration of a synthetic antagonist of folic acid. More than any of the other vitamins, pteroylglutamic acid and its close chemical relatives, the "folic acid family," are concerned with the formation of blood cells in animals.

PTEROYLGLUTAMIC ACID IN THE CLINICAL TREATMENT OF THE ANEMIAS

A characteristic hematological response is obtained when pteroylglutamic acid is administered to patients with pernicious anemia, sprue, or the megaloblastic anemias of infancy, pregnancy or pellagra. The usual dosage is 5 to 10 mg. daily either orally or by injection, although smaller amounts have in many cases been found to be sufficient.

During 1946 pteroylglutamic acid was made available for experimental use before being introduced commercially and it was noted that the neurological signs and symptoms associated with pernicious anemia were not usually alleviated by pteroylglutamic acid,[40, 41] although these were known often to respond to liver extract. Consequently when pteroylglutamic acid was introduced, a warning was given not to use the substance alone in the treatment of pernicious anemia, but to continue the use of liver extract in this disease. The administration of pteroylglutamic acid alone to patients with pernicious anemia was practiced by a number of investigators who noted the aggravation or initiation of cord changes under this regimen and free speculation arose as to the etiology of these changes. It was actually suggested, despite a lack of biochemical evidence, that pteroylglutamic acid was an antagonist of glutamic acid, and as such had an injurious effect.[42] An experimental examination of this suggestion showed it to be without foundation.[43] A more obvious and simple explanation of the neurological disturbances was that the patients needed the active factor, vitamin B_{12}, of concentrated liver extract.[44] This explanation is sufficient to account for the observed facts and is adequately supported by the finding that vitamin B_{12} will clear up the neurological changes in a patient with pernicious anemia when these changes have appeared during a course of treatment with pteroylglu-

tamic acid alone and when the administration of pteroylglutamic acid is continued concurrently with vitamin B_{12}.[45]

An interesting antithesis is provided by certain observations made in puerperal megaloblastic anemia. This condition may be exacerbated by the administration of vitamin B_{12} but yields to pteroylglutamic acid.[46, 47]

Speculation arose as to the possibility that pernicious anemia and sprue might be caused or aggravated by a failure in the normal hydrolysis of pteroylglutamic acid conjugate and a consequent inability to utilize dietary sources of pteroylglutamic acid.[48, 49] No increase in urinary pteroylglutamic acid was observed in patients with pernicious anemia following the administration of the conjugate, although an increase occurred in normal subjects. It was suggested that a deficiency of pteroylglutamic acid occurred in the patients as a result of their inability to utilize the conjugate and that the hemopoietic mechanism was slowed as a consequence of the deficiency. Three patients with pernicious anemia and one with macrocytic anemia following gastrectomy were treated for eight to 12 days with an oral dose of the conjugate equivalent to 2.3 to 4 mg. of pteroylglutamic acid daily.[48] No responses were produced, but when equivalent amounts of pteroylglutamic acid were substituted for the conjugate all of the patients showed significant therapeutic responses. In another investigation,[49] the daily oral administration of 1 mg. of conjugate for 10 days, followed by a second period of 11 days in which the conjugate was given with 100 ml. of normal human gastric juice, produced no response in a patient with pernicious anemia. The patient then responded submaximally to a daily dose of 0.35 mg. of pteroylglutamic acid. A second patient was given intramuscular injections of 2.5 mg. of conjugate daily for 12 days followed by a single injection of 30 mg. without responding. A maximal reticulocyte

response was produced 12 days later by administering pteroylglutamic acid. Suggestions were advanced that the action of the anti-pernicious-anemia factor of liver extract might be concerned with the liberation of pteroylglutamic acid from the conjugate [50] or with the removal of an inhibitor of the conjugase system.[51] Subsequent investigations led to modifications of these conclusions, for responses in patients with pernicious anemia were obtained to concentrates of the conjugate [52] and liver extract was not found to increase the urinary excretion of pteroylglutamic acid following administration of the conjugate in two patients.[53] The proposal that pernicious anemia is due to a failure to hydrolyze dietary pteroylglutamic acid conjugate has received little support or attention in the scientific literature subsequently to 1947.

A concentrate of the conjugate was effective in a case of sprue [52] and a patient with "nutritional macrocytic anemia" responded to the conjugate.[54]

Pteroyl-γ-glutamyl-γ-glutamyl-glutamic acid ("pteroyltriglutamic acid," "Teropterin") is another naturally-occurring substance which is hydrolyzed by conjugase. Pteroyltriglutamic acid has been found to produce hemopoietic responses readily in pernicious anemia and other megaloblastic anemias.[52, 55, 56]

OCCURRENCE OF PTEROYLGLUTAMIC ACID IN BIOLOGICAL MATERIALS

Biological tests of natural materials have revealed the presence of pteroylglutamic acid in most animal and plant tissues. The amounts present are too small to estimate by chemical analysis, and microbiological assays for folic acid activity must be used. The two organisms most commonly employed are *Streptococcus faecalis* R and *Lactobacillus*

casei. For the estimation of the total content it is necessary to treat the samples with a preparation of "conjugase," because the vitamin occurs in nature partly or largely in the form of the conjugate to which these organisms show very little response. Kidder has found that the protozoon *Tetrahymena geleii* W can utilize either the conjugate or free pteroylglutamic acid equally well[57] so that it may be possible to use this organism for the assay of pteroylglutamic acid without a preliminary treatment of the samples with conjugase.

In some of the earlier investigations,[58, 59] "taka-diastase" was used to liberate pteroylglutamic acid from natural materials, but Simpson and Schweigert[60] found that taka-diastase contains the conjugate from which pteroylglutamic acid may be liberated by conjugase present in the materials which are being assayed.

When carried out by any of the accepted methods, the biological assay for folic acid activity will give a value which will include the content of any citrovorum factor (CF) which may be present in the sample in addition to pteroylglutamic acid. The test organisms which are used in the assay for pteroylglutamic acid respond also to CF. The sample may be assayed also with *Le. citrovorum* to determine its CF content, and the pteroylglutamic acid content may then be calculated from the formula:

> Folic acid activity (calculated as pteroylglutamic acid by comparison with standard curve) = Pteroylglutamic acid plus 0.5 CF (as leucovorin).

If the presence of pteroic acid or formylpteroic acid is suspected, a further correction may be made by assaying the sample with *L. casei* for which these compounds are inert (Table 3.1). However, they are not known to be of common occurrence.

The pteroylglutamic acid content of feedstuffs may also be measured by assay with chicks on a purified deficient diet. A growth response is obtained which is proportional within a certain range of the pteroylglutamic acid content of the diet. Some results obtained by this method are shown in Table 3.2.*

METABOLISM OF PTEROYLGLUTAMIC ACID

Only small amounts of folic acid activity, less than 1 per cent of the probable dietary intake, were found to be excreted in the urine of human subjects.[36, 62] Oral or parenteral administration of pteroylglutamic acid was found to produce an excretion of 15 to 75 per cent of the dose within 24 hours, depending upon the size of the dose.[52] Other investigators reported similar findings.[64, 65]

Further studies [66] showed the excretion of folic acid activity, as measured by assay with *S. faecalis* R, was almost as high following oral dosage with sodium pteroylglutamate as following intravenous injection (Table 3.3). An average of 90 per cent of the 24 hour excretion took place in the first 6 hours following oral dosage with 5 mg. of the sodium salt; after 24 hours the urinary content of folic acid activity returned to the basal level of between 5 and 10 millimicrograms per ml.

Similar time relationships were described by Denko [67] who found that the urinary excretion following oral dosage rose to a peak in one to four hours and then dropped gradually to normal levels within 24 hours. Usually 35 to 75 per cent of the injected dose of 5 mg. was excreted in the urine in 24 hours.

* Agricultural Handbook No. 29, *Folic Acid Content of Foods* (U.S. Government Printing Office, September, 1951) which contains an exhaustive compilation of data.

TABLE 3.2

Folic Acid Activity of Various Natural Materials Expressed in Terms of Pteroylglutamic Acid as Parts Per Million[61]

Material	*Content*		
	(*a*)	(*b*)	(*c*)
Yellow corn	0.3	0.29	0.23
Oats	0.2	0.40	0.34
Barley	0.6	0.49	0.41
Wheat	0.4	0.40, 0.62	0.55
Wheat bran	2.1	2.40	1.60
Wheat middlings	0.9	1 80	1.40
Wheat germ			2.80
Alfalfa meal and leaf meal	6 to 11		5.00
Alfalfa hay		3.00	2.40
Dried turnip greens	14.0	5.90	6.20
Mustard greens		9.30	9.40
Swiss chard		0.70	0.62
Spinach, fresh		2.80, 0.5	2.20
Spinach, dried		22.00	19.00
Soybean meal	7.7		
Soybeans		5.30	3.60
Soybean flour	4.0	4.40	4.10
Cottonseed meal	2.3		
Corn gluten meal	0.2		
Linseed meal	3.2		
Beef round, fresh		0.07	0.04
Lean pork, fresh		0.09	0.09
Fish meal	0		
Dried whey	0.9		
Tankage	1.6		
Dried egg yolk		0.50	
Beef liver, fresh		4.00	
Dried pork liver		19.00	15.00
Dried yeast		1.40 to 6.20	1.40 to 6.00
Dried yeast		8.80	
Leaf lettuce		0.84	0.69
Endive		0.75	0.62
Kale		0.92	1.00
Parsley		1.70	1.70
Asparagus		1.24	1.18
Broccoli		0.90	1.10
Beets			0.42
Green beans		0.71	0.71
Peas		0.23	0.22
Radishes		0.13	0.11
Tomatoes		0.14	0.12

(a) Chick assay[63] (b) *S. faecalis* assay (c) *L. casei* assay

TABLE 3.3

Effect of Dosage and Mode of Administration on Urinary Excretion of Pteroylglutamic Acid

Form Administered	*Amount PGA Taken (Mg.)*	*Collection Period of Urine (Hr.)*	*Route of Administration*	*Per Cent of Dose Excreted in Urine*	*Average*
Solution of Na salt	0.1	6	Oral	0, 0, 0.9, 0.6	0.4
Solution of Na salt	0.5	6	Oral	0, 12, 25, 19, 3, 14	12.0
Solution of Na salt	1.0	6	Oral	10, 25, 26, 26	22.0
Solution of Na salt	2.0	6	Oral	32, 36, 38, 51, 56	43.0
Solution of Na salt	5.0	6	Oral	45, 49, 49, 44, 48, 52, 48, 46, 57, 56	49.0
Solution of Na salt	15.0	24	Oral	81, 92, 57	77.0
Solution of Na salt	5.0	6	Intravenous	51, 54, 72, 64	60.0
Suspension in water	5.0	6	Oral	35, 31, 39, 32, 35	34.0
Suspension in oil*	5.0	6	Oral	15, 15, 17, 24, 28	20.0

*Mazola oil.

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CHAPTER IV

THE VITAMIN B_{12} GROUP OF COMPOUNDS

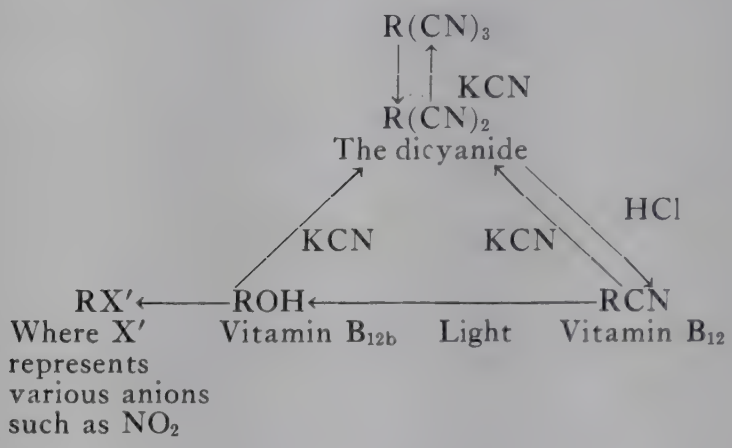
CHEMISTRY

A CRYSTALLINE substance, "vitamin B_{12}," with the activity of the anti-pernicious-anemia factor of concentrated liver extracts was isolated from liver by Rickes and co-workers in the U. S. A. and independently by Lester Smith in England,[1, 2] both in the spring of 1948. A second equally active substance, vitamin B_{12b}, was shown to be present in liver extract and was crystallized from *Streptomyces aureofaciens* fermentations.[3] The name "vitamin B_{12c}" was applied to a third compound which was later shown to be a nitroso derivative.[4]

Interest in vitamin B_{12} was heightened by the disclosure that the compound was unique among organic substances of biological origin in containing cobalt.[5, 6] The molecule was found to contain one atom of cobalt and one atom of phosphorus and the cobalt remained in firm combination even after the substance had been extensively broken down by acid.[7]

The relationships between the compounds in the vitamin B_{12} group were clarified by a series of publications which appeared in 1950 and which showed that the group was a series which consisted of the same parent molecule coordinated with various anionic groups.[8, 9, 10, 11] The formation of a purple compound by treating vitamin B_{12} with

potassium cyanide was described.[10] The compound reverted to vitamin B_{12} upon treatment with dilute hydrochloric acid. When vitamin B_{12b} was similarly treated, the purple compound was again formed and vitamin B_{12} was produced by hydrolysis with dilute acid.[11] Vitamin B_{12} may be converted to vitamin B_{12b} by photolysis.[9] Complexes containing 2 or 3 mols of cyanide may be formed.[15] These changes may be represented as follows:



Photomicrographs of crystalline vitamins B_{12} and B_{12b} are shown in Figures 4.1 and 4.2.

A preliminary description of the results of acid hydrolysis of vitamin B_{12} was made by Ellis, Petrow and Snook [7] who noted the presence of a ninhydrin-reacting substance in such hydrolysates. An almost black, amorphous, cobalt-containing fragment was also obtained which upon acidification was soluble in ether, chloroform and acetone. The Merck group [12] reported a specific rotation of $(\alpha)^{23}{}_{6563} = -59^\circ \pm 9^\circ$ and a composition typified by $C_{61\text{-}64}H_{86\text{-}92}N_{14}O_{13}P$ Co for vitamin B_{12}. Alkali fusion formed products which gave a red color with *p*-dimethylaminobenzaldehyde, characteristic of pyrroles and other cyclic 5-membered nitrogenous compounds. The investigators concluded that the product of acid hydrolysis which reacted with ninhydrin and which was described by Ellis

Figure 4.1 Photomicrograph of crystalline vitamin B_{12} obtained from *Streptomyces aureofaciens* fermentation.[3]

Figure 4.2. Photomicrograph of crystalline vitamin B_{12b} obtained from *Streptomyces aureofaciens* fermentation.[3]

et al.[7] appeared to be due to residual impurities. This conclusion was later shown to be erroneous and the fragment was identified as D-1-amino-2-propanol,[13] two groups per vitamin B_{12} molecule,[10, 14] after a preliminary inference that the fragment was 2-aminopropanol [16] which was later withdrawn.[17]

Acid hydrolysates of vitamin B_{12} were shown [18] to contain three other fragments: (a) 5,6-dimethylbenzimidazole; [18, 19] (b) its 1-α-D-ribofuranoside; [20, 21] and (c) the monophosphoric ester of (b).[22] Doubtless a and b are formed by acid hydrolysis of c.

According to present information, the molecule of vitamin B_{12} apparently consists of 5,6-dimethylbenzimidazole-1-α-D-ribofuranoside-3-phosphate [21] which is esterified through the phosphate group to a cobalt complex which contains two 1-amino-2-propanol groups and a coordinately-linked cyanide group which is easily replaceable by other anions without change in biological activity. A discussion of the electron arrangements in covalent complexes of Co^{+++} was presented by Cooley and co-workers [23] who drew attention to the close analogy between Fe^{++} and Co^{+++} indicating that analogies might be drawn between transformations of the vitamin B_{12} group and those of the ferroporphyrins. They described a reaction between vitamin B_{12} and liquid ammonia which led to the production of "ammonia cobalichrome," a biologically active analogue of vitamin B_{12}.

VITAMINS B_{12a} AND B_{12b}

The name "vitamin B_{12a}" was applied to a crystalline material which was obtained from vitamin B_{12} by catalytic hydrogenation followed by oxidation with air. Vitamin B_{12a} had absorption spectrum maxima at 315, 352.5, 415 and 530 mμ and biological activity for *L. lactis*, *L. leich-*

mannii, rats and chicks which was much lower than that of vitamin B_{12}.[24] Soon afterwards the isolation of crystalline vitamin B_{12b} from *Streptomyces aureofaciens* was reported.[3] The absence of a band in the absorption spectrum at 315 mμ served to differentiate it from vitamin B_{12a}; furthermore, vitamin B_{12b} was just as active as vitamin B_{12} in assays with chicks and *L. leichmannii* in contrast to vitamin B_{12a}.[25] It was found by Brockman and co-workers [26] that a compound having all the known characteristics of vitamin B_{12b} rather than of vitamin B_{12a} was formed when vitamin B_{12} was hydrogenated. The Merck group then reported [27] that their previous biological data for vitamin B_{12a} were "not intended as a critical and final evaluation" and one must conclude that these data were erroneous. They stated that the absorption band at 315 mμ could be produced by heating the compound at 100° for two hours *in vacuo*, and then dissolving in water and reading the absorption spectrum immediately. Upon standing at room temperature, the absorption spectrum became identical with that of vitamin B_{12b}. It appears from their observations that "vitamin B_{12a}" may be an anhydro-form of vitamin B_{12b}.

Comparatively mild chemical treatments of vitamin B_{12}, such as standing in dilute hydrochloric acid at room temperature, were shown by Brockman and co-workers [28] to produce a vitamin B_{12b}-like absorption spectrum, and it was found by Veer and co-workers [9] that vitamin B_{12} could be changed to vitamin B_{12b} by exposure to diffuse daylight for a few hours in dilute acidic solution.

THE BIOLOGICAL FORMATION OF VITAMIN B_{12} AND ITS DISTRIBUTION IN NATURAL MATERIALS

Vitamin B_{12}, like the other B-vitamins, is formed by bacteria in the digestive tract. One of the first indications of this was found by Hammond [29] who reported that "dried

cow manure and fish meal appear to contain a nutrient, not supplied in adequate quantity by the other ingredients of the diets, that is required for rapid growth and high efficiency of feed utilization. The results . . . indicate that dried rumen contents also contain this nutrient. Mortality was consistently lowest in the pens of chicks that received dried cow manure or dried rumen contents in their diet." This nutrient, which showed its effects in chicks on "all-vegetable" diets, is now known to be vitamin B_{12}. The presence of the nutrient in the feces of hens was shown by Rubin and co-workers [30] who concluded that it was synthesized in the lower portion of the digestive tract or in the voided feces. Stokstad and co-workers [31] prepared active concentrates from fermentation materials produced by a nonmotile rod-shaped organism which was isolated from hen feces. The concentrates promoted the growth of chicks on a corn-soybean meal diet and were effective when injected into patients with pernicious anemia. A few months later Rickes and co-workers [32] reported the isolation of crystalline vitamin B_{12} from culture broths of *Streptomyces griseus* and the production of activity for *L. lactis* Dorner by several microorganisms.

Thus by the end of 1948 a series of investigations had laid the foundation for a new industry, the production of vitamin B_{12} by controlled fermentation. By an unexpected turn of events, the aureomycin [3] and streptomycin [32] fermentations, in which *Streptomyces* organisms are used, were found to yield large quantities of vitamin B_{12} as a by-product. The new material almost immediately found its way into animal feeding and pharmaceutical channels. The shortage of vitamin B_{12} in poultry foods and the dependence of the feed industry on animal products as sources of the vitamin were soon to come to an end.

The use of primary fermentation for the large-scale

production of vitamin B_{12} was advocated by Lewis and co-workers [33] who described the use of *B. megatherium* for this purpose. Yields of 0.8 parts of vitamin B_{12} activity per million of whole culture medium were produced in 12 hours in a medium containing sucrose, yeast extract, citric acid, ammonium and inorganic salts. Halbrook and co-workers [34] found that biological activity for *L. leichmannii* was produced by various microbial colonies obtained from culturing poultry-house litter and chicken feces. One active organism was tentatively identified as a strain of *Aerobacter aerogenes*.

The presence of vitamins in human feces has frequently been noted, and the coprophagy which makes such important contributions to animal nutrition has perhaps functioned similarly in primitive peoples. McGee has described the preservation, drying and ingestion of feces by the Seri Indians of Tiburon Island in the Gulf of Mexico, who think that endurance for the hard warpath or prolonged chase is derived from consumption of the dried excrement.[35]

Vitamin-B_{12}-like activity was found present in the stools of a patient with pernicious anemia by Bethell and co-workers.[36] A hemopoietic response was produced in a patient with pernicious anemia by injecting an extract made from the feces of a second patient.[37] Callender and co-workers [38] have also reported the presence of vitamin B_{12}-like activity for *L. lactis* in the feces of patients with pernicious anemia. Since such patients have little or no ability to utilize vitamn B_{12} from oral sources, it was not possible to draw conclusions from these reports as to whether the fecal vitamin B_{12} was of alimentary or bacterial origin in these subjects. However, Girdwood [37] found that the contents and secretions of the small intestine in patients with pernicious anemia contained negligible amounts of vitamin B_{12} activity but about 5 micrograms

were found daily in the feces, perhaps indicating synthesis in the large bowel.

An extensive study of source materials was made by Robbins and co-workers [39] who used assays with *Euglena gracilis*, and employed both turbidimetric and growth-zone procedures. No vitamin B_{12} was found in ungerminated barley, yellow corn meal, toasted wheat germ, carrots, parsnip and sweet potato. Negative results were obtained for the fruits of green pepper, string beans, peas, banana, avocado, coconut, grapes and persimmon; also for the germinated seeds of barley, stringbeans, soybeans, corn, peas, radish, rutabaga, Swiss chard and wheat. Yeast contained only 2 to 4 micrograms of vitamin B_{12} per kilogram of dry material. Seventy-six out of 88 Actinomycetes isolated from soil were found to produce vitamin B_{12} activity when grown in shake cultures, the maximum amount being 0.5 microgram per ml. The authors concluded that the synthetic activity of microorganisms, especially bacteria and Actinomycetes, and not that of higher plants, is the original major source of vitamin B_{12} in nature. Herbivorous animals apparently obtain vitamin B_{12} from the microflora of their digestive tracts, and this would account for the presence of the vitamin in the liver of cattle from which extracts are prepared for medicinal use. The vitamin is found in the internal organs and muscular tissues of vertebrates. Some information on the distribution of vitamin B_{12} is in Table 4.1.

VITAMIN B_{12} IN THE TREATMENT OF PERNICIOUS ANEMIA

Twenty years of widespread and intensive study of the effects of liver extract upon pernicious anemia had served to set the stage and rehearse the clinical scene for the ap-

TABLE 4.1

Vitamin B_{12} Content of Natural Materials

Reference	*Material*	*Vitamin B_{12} Micrograms Per Gram Fresh Material*	
		Root Stem	*Leaf*
a	Tomato	0.015 0.002	0 in 0.26 gram
a	Cabbage	0.01 0.005	0 in 0.2 gram
a	Celery	0.002 . . .	0 in 0.6 gram
a	Pepper, green	0.002 0 in 0.4	0 in 0.4 gram
a	Kale	0.01 0.0002	0 in 0.2 gram
a	Kohlrabi	0.01γ 0 in 0.6 gram	Less than 0.0001 in 4 gram
a	Broccoli	0.01 0 in 0.5 gram	0 in 0.56 gram
a	Leek	0.01 0 in 0.6 gram	0 in 0.6 gram
		Per kilo or liter of fresh material	
a	Soil	2 to 15	
a	Cows' milk	1 to 2	
a	Roots of various garden vegetables	4 to 10	
a	Excised tomato roots grown in Pfeffer's solution	None	
a	Pond water	1 to 20	
		Micrograms per gram of solids	
b	Hog liver	1.8	
b	Calf liver	2.4	
b	Liver residue from hot water extraction	0.3	
b	Beef round	0.18	
b	Starfish	0.18	
b	Oyster	2.8	
b	*Artemia salina* eggs	7.2	
b	Winkle	1.2	
b	Sand worm	5	
b	Earth worm	1.1	
c	Ling cod liver	0.43 to 0.88	
c	Ling cod muscle	0.18	
c	Halibut liver	0.52	
c	Dogfish liver	0.1	
c	Sockeye salmon liver	1.7	
c	Sockeye salmon kidney	18.0	
c	Sockeye salmon pyloric ceca	3.1	
c	Sockeye salmon spleen	7.2	
c	Sockeye salmon milt	0.48	
c	Sockeye salmon eggs	1.7	
c	Sockeye salmon stomach	1.7	
c	Red cod muscle	0.12	

TABLE 4.1—*continued*

		Micrograms per gram of solids	
c	Condensed fish solubles	0.02 to 0.67	
c	Fish meals	0.17 to 1.5	
c	Meat meal	0.17	
c	Little neck clams	2.5	
		Per 100 Grams Dry Weight	
		Rat Assay	*Microbiological Assay*
d	Beef round (cooked)	5.5	5.0, 7.9
d	Beef tongue (cooked)	5.5	7.6
d	Pork shoulder (cooked)	0.9	0.7, 3.0
d	Pork ham (cooked)	2.2	2.9, 3.0
d	Veal	3.6	3.0
d	Horsemeat	7.5	7.0
d	Fish solubles	40	15, 25
d	Beef liver	50	47, 50
d	Tomato juice	0	0
d	Beef kidney		50
d	Mutton (fresh)		8.8
d	Beef heart		25
d	Hog spleen		0, 9, 22
d	Hog adrenal		15
d	Crude casein		3, 7
d	Cows' milk		2
d	Cheddar cheese		2.5
d	Egg yolk		3
d	Sheep rumen contents		100
d	Guinea pig feces		230
d	Chicken manure		450
d	Goat manure		20
d	Cow manure		47
d	Chicken breast (fresh)		5.3
d	Chicken leg (fresh)		5.2
d	Salmon		8.5
d	Oysters		15

[a]Euglena assay, Robbins, W. J., Hervey, A., and Stebbins, M. E.[39]
[b]Rat assay, Zucker, T. F., and Zucker, L. M.[40]
[c]L. leichmannii assay, Tarr, H. L. A., Southcott, B. A., and Ney, P. W.[41]
[d]Elvehjem, C. A.[42]

pearance of vitamin B_{12} in 1948. The crystalline vitamin was promptly found to duplicate the effects of concentrated liver extracts in producing a hemopoietic remission and an amelioration of the neurological symptoms and glossitis

in pernicious anemia.[2, 43, 44, 45, 46, 47] The question of the possible existence of therapeutic quantities of factors other than vitamin B_{12} in such extracts remains to be explored. Just as in the case of liver extracts, vitamin B_{12} was shown to be of very low effectiveness when administered orally unless given in amounts many times greater than the liminally effective parenteral dose or unless normal human gastric juice or some other source of the "intrinsic factor" was simultaneously fed.[48, 49]

Vitamin B_{12} is remarkable for the smallness of the amount required to bring about a remission in pernicious anemia. A daily injection of from 1 to 3 micrograms daily produced a typical response.[43, 50] The average amount of vitamin B_{12} per U.S.P. unit of liver extract as measured by microbiological assay with *L. lactis* Dorner was even less, only 0.1 to 0.9 microgram [1] but partial destruction may have occurred during the preparation of the samples for assay.[51, 52] Most investigators find that a satisfactory hemopoietic response is obtained with 1 to 2 micrograms of vitamin B_{12} injected daily, although some cases have responded to smaller amounts. The requirement of vitamin B_{12} per unit of body weight is considerably less in the case of patients with pernicious anemia than in animal nutrition.

The first announcement of the effects of vitamin B_{12} in pernicious anemia was made by West who found that three patients showed rises in reticulocytes, red cell count and hemoglobin following the administration of vitamin B_{12}.[43] Two patients received 6 and 150 micrograms of vitamin B_{12} respectively and the third was given 3 micrograms followed by 50 micrograms. Indications soon followed that the vitamin was effective against the spinal cord symptoms of combined system disease,[2, 45, 46, 47, 53] although the definite establishment of this point necessitated more prolonged study.

Sensory and motor disturbances which are associated with changes in the posterior columns and pyramidal tracts of the spinal cord may occur in untreated or relapsed cases of pernicious anemia. These involvements may progress to an irreversible state if untreated, but generous dosage with concentrated liver extract will produce an improvement in the symptoms as well as causing hemopoietic response if treatment is initiated promptly. Similar improvements are obtained by injecting vitamin B_{12}. It was reported by Mueller and co-workers [54] that 5 micrograms on alternate days produced a rapid response in cases in which the neurological symptoms were of recent origin, but this dosage had little or no effect in a patient who had suffered from cord lesions for more than two years. A daily injection of 10 micrograms for combined system disease was suggested. No response to an average weekly dose of 40 to 60 micrograms of vitamin B_{12} was found when the nerve damage had a duration of more than 18 months.[55] The use of large doses of vitamin B_{12} in the treatment of the neurologic lesions was advocated by Hall and co-workers.[56]

The effects of varying the amount and frequency of the dosage of vitamin B_{12} in pernicious anemia have been widely explored and the results have been very similar to those obtained with liver extract in that a great deal of variability exists in the time between the cessation of therapy and the onset of relapse. Mueller and co-workers [54] suggest that relapsed pernicious anemia should be treated with 5 micrograms daily for 10 days and 5 micrograms weekly thereafter. They recommend that the maintenance dose be individually determined for each patient. The effects of a single injection were studied by Erf and Weiner [57] who found that the injection of 50 to 100 micrograms of vitamin B_{12} produced a remission lasting from 50 to 100 days and that the administration of 50 micrograms to a patient already

in remission would prolong the remission for 70 to 120 days. These results are similar to previous findings with liver extract. The storage of vitamin B_{12} in the tissues evidently may be excellent, far exceeding the period of retention observed for other B-complex factors such as thiamine.

Single injections were used by Ungley who found [58] that there was little or no hemopoietic response to 1.25 micrograms or less, a good response to 10 micrograms and an increased response to larger doses up to 80 micrograms. A satisfactory maintenance of 18 out of 21 patients who were followed from six to 15 months was obtained with 10 micrograms every two weeks. However, the remaining three patients showed glossitis and one of them occasionally had macrocytosis. Bethell maintained a series of 20 patients in remission for 18 months by the use of an equivalent of 1 microgram daily.[59] A single injection of 25 micrograms was reported by Beard and co-workers [60] to produce a response lasting for about 25 days in two cases and the response to 50 micrograms lasted about 55 days in two other cases. Bone marrow changes were noted only six hours after the injection of 100 micrograms.

Oral doses of 3 milligrams of vitamin B_{12} were found by Ungley [61] to produce excellent responses in pernicious anemia and he concluded that in the five cases studied the responses were as great as any that had been obtained with injected vitamin B_{12}. These results suggest that a threshold for the uptake of vitamin B_{12} from the intestine may exist in pernicious anemia and that the dosage employed was sufficient to exceed the threshold.

Vitamin B_{12b} was found to be effective parenterally in the treatment of patients with pernicious anemia in amounts of 1 to 2 micrograms daily.[62] Similar results were reported by Fricke and co-workers [63] who concluded that vitamin B_{12b} was clinically as effective as vitamin B_{12}.

Remissions in two cases of pernicious anemia treated with thymidine were described by Hausmann.[64] The preparation used, a concentrate containing 85 to 90 per cent of thymidine, was prepared from purified anti-pernicious-anemia liver extract and was administered at the rate of 200 to 280 milligrams daily. The criterion used for the absence of vitamin B_{12} was a negative test with α-nitroso β-naphthol. Since a contamination with one part of vitamin B_{12} in 200,000 of the preparation would have been sufficient to account for the clinical responses, one must conclude that a more delicate test for the absence of vitamin B_{12} would have been preferable. Other investigators have used thymidine in single doses of respectively 5.3 mg.,[65] 48 mg.,[66] and 150 mg.[67] with negative or insignificant responses in pernicious anemia.

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CHAPTER V

THE INTRINSIC FACTOR

Soon after the therapeutic effect of liver on pernicious anemia was noted, it was found that two substances appeared to be involved in producing remissions. One, the "extrinsic factor," was present in foods typified by lean beef, and the other, the "intrinsic factor," was present in normal gastric juice.[1, 2]

The feeding of lean beaf did not result in remission in patients with pernicious anemia. However, when the beef was first fed to a normal subject and was then recovered from his stomach after partial digestion, the resultant material had therapeutic potency when administered orally to such patients. No activity was present in beef which had been exposed to digestion in the stomach of a person with pernicious anemia. These experiments together with later investigations showed that pernicious anemia is a deficiency disease which is caused by the lack of a substance which is present in normal gastric secretions but which is not formed in sufficient quantity by those suffering from the disease. This substance, the "intrinsic factor" is needed for the uptake of the "extrinsic factor," now presumed to be vitamin B_{12}, from the gut.

The intrinsic factor is always present in gastric juice if free hydrochloric acid is secreted, however, the factor may be present even if hydrochloric acid is absent. The factor is

easily destroyed by heat, and the potency of a mixture of lean beef with gastric juice is rapidly destroyed by exposing it to boiling temperatures showing that no thermostable compound is formed *in vitro* by incubating the mixture. The intrinsic factor is present in the mucosa of the small intestine of pigs.

The relationship between vitamin B_{12} and the intrinsic factor was studied by several groups of investigators. Daily doses of 5 or 10 micrograms of B_{12} were found to be ineffective when given by mouth unless a source of the intrinsic factor such as normal gastric juice was given simultaneously.[3] This indicated that vitamin B_{12} had the properties of the extrinsic factor. Similar observations were reported by Hall and co-workers in 1949 [4] who found that normal human gastric juice after sterile filtration had the property of producing a remission in pernicious anemia when given orally together with vitamin B_{12}. The latter substance was ineffective when given by mouth without gastric juice.

Ungley [5] studied patients who received oral doses of vitamin B_{12} together with normal gastric juice and by measuring the quantitative hemopoietic response he attempted to calculate the amount of the vitamin which had been absorbed. He concluded that each 100 ml. of gastric juice seemed to be enough for the absorption of 0, less than 1, 1, less than 2, 5 or 10, 10, 10, and 10 micrograms of vitamin B_{12} respectively in a series of eight patients. Thus the amount of gastric juice necessary to promote the absorption of a given amount of vitamin B_{12} varied considerably, perhaps due partly to differences in the amount of intrinsic factor in the samples of gastric juice and partly to differences in the recipients. Studies were also made [6] on the introduction of vitamin B_{12} through a Miller-Abbott tube into a washed segment of intestine isolated between two

balloons. Little or no response was produced to 40 or 80 micrograms of vitamin B_{12} with or without gastric juice, but the author pointed out that the segment of intestine may have been too small or in an area unsuitable for absorption of vitamin B_{12}. No response was obtained when a single oral dose of 80 micrograms of vitamin B_{12} was given to a patient who had received a preliminary course of phthalylsulfathiazole, aureomycin and dihydrostreptomycin by mouth for six days.

Further studies on the oral administration of vitamin B_{12} were reported by Ungley.[7] He found that only poor responses were obtained when 18 micrograms of vitamin B_{12} per day was given by mouth for 24 days. The increase in erythrocyte count was less than would have been expected in 15 days from a single injection of 5 micrograms. The subsequent daily administration of 1 microgram by injection was followed by a satisfactory increase in erythrocytes. These observations strikingly emphasize the lack of uptake of vitamin B_{12} from the digestive tract which may occur in pernicious anemia. In another case the daily administration of 5 micrograms of vitamin B_{12} was ineffective whereas the same quantity given daily for 10 days with 50 ml. of normal unfiltered gastric juice produced a response equivalent to that expected from a single dose of 10 micrograms by injection. Filtration of the gastric juice through a Seitz filter led to loss of activity of intrinsic factor. The author suggested from these and similar findings that vitamin B_{12} given by mouth with gastric juice may be from one-fifth to four-fifths as effective as the same dose of vitamin B_{12} given by injection. This may be compared with the ratio of effectiveness between oral and injected vitamin B_{12} for normal chicks which was calculated by Stokstad and co-workers [8] to be in the neighborhood of 1:2.

A suggestion to explain the mechanism of the action of

intrinsic factor was made by Ternberg and Eakin.[9] They observed that a vitamin B_{12}-binding effect was shown by normal human gastric juice and by extracts of mucosa of the stomach or of the small intestine of the pig, all of which materials are known to have intrinsic factor activity. The vitamin B_{12}-binding effect was measured by assay with *Escherichia coli* in a culture medium containing sulfanilamide. This medium inhibits growth of the organism but the addition of vitamin B_{12} enables growth to take place. The effect of vitamin B_{12} was found to be abolished by the simultaneous addition of unheated protein fractions from gastric juice. It was concluded that these fractions contained a non-dialyzable heat-labile substance termed "Apoerythein" which formed a complex with vitamin B_{12}, thus rendering the vitamin unavailable to *E. coli*. The authors suggested that apoerythein was probably identical with the intrinsic factor or was an important component of it. Other investigators reported that extracts of this type showed vitamin B_{12}-binding activity in the *L. leichmannii* assay. The binding-effect was destroyed by heating.[10, 11, 12] No clinical results with apoerythein were reported by the Texas group but Prusoff and co-workers found that fractionation of solutions obtained from hog gastric mucosa led to separation of the clinically measured intrinsic factor activity from most of the vitamin B_{12}-binding action. These investigators used saline extracts and prepared fractions by adding increasing amounts of ammonium sulfate. Three principal fractions were prepared: (a) Zero to 35 per cent saturation; (b) 35 to 55 per cent; and (c) 55 to 100 per cent. The last fraction contained less intrinsic factor activity than fraction b, however, fraction c showed the greatest B_{12}-binding activity as measured microbiologically.

Further speculations upon the nature and role of "apo-

erythein" were made by the Texas investigators.[13] Vitamin B_{12}-binding activity was found to be present in the saliva of both normal persons and pernicious anemia patients in amounts sufficient to account for that found in the gastric juice. The gastric juice of the patients was stated to contain a principle which inactivated the vitamin B_{12}-binding activity unless the gastric juice was first treated with hydrochloric acid. The theory was advanced that the primary defect in pernicious anemia is the achlorhydria which prevents inactivation of the destructive principle in gastric juice. In terms of this theory, the intrinsic factor is produced by pernicious anemia patients in normal amounts but is destroyed in their stomachs because of the absence of hydrochloric acid. There are various obvious objections to this theory; achlorhydria is far more common than pernicious anemia; the administration of hydrochloric acid has not been shown to produce a remission in pernicious anemia; gastrectomy often results in a pernicious anemia-like condition, and vitamin B_{12}-binding activity has not been shown to be coincident with intrinsic factor activity.

Lysozyme was found by Meyer and co-workers to render vitamin B_{12} unavailable to *L. lactis* Dorner and to *E. coli*, however, lysozyme was not able to function as "intrinsic factor" in clinical tests.[11] Bird and Hoevet[14] found that concentrates of "intrinsic factor" bound vitamin B_{12} but did not bind thymidine as shown by their effect in the assay with *L. leichmannii;* moreover vitamin B_{12} was dialyzable from a mixture with lysozyme but not from the complex formed by combination of the vitamin with the preparation containing the intrinsic factor.

A relationship between intrinsic factor and gastric mucoprotein from human sources was postulated by Glass and co-workers.[15] However the dosage of this material needed to produce a response was in the neighborhood

of 50 to 100 mg., which was considerably greater than the effective dose of Prusoff's fraction.[12]

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CHAPTER VI

ANTAGONISTS OF PTEROYLGLUTAMIC ACID

By the time it was synthesized, pteroylglutamic acid had been shown to be closely related to certain normal processes connected with cellular proliferation, as follows:

1. "Megaloblastic arrest" and hypocellularity in the bone marrow with reductions in the numbers of erythrocytes, leucocytes and thrombocytes in the circulating blood are produced in certain species by a dietary deficiency of pteroylglutamic acid and are corrected by its administration.

2. Pteroylglutamic acid was the first vitamin which was shown to be specifically related to the formation of a fragment of desoxyribonucleic acid. It was found that either thymine or thymidine would replace pteroylglutamic acid as a growth factor for *Streptococcus faecalis* R.

Such considerations made it evident that if it were possible to produce a biologically antagonistic analog of pteroylglutamic acid by synthesis, the compound might have important effects in arresting the formation of new cells. These anticipations were first realized in 1947. The first successful approach to synthesizing a "folic acid antagonist" was reported by Martin, Tolman and Moss[1] and independently by Hultquist and Smith.[2] The reaction was carried out in a manner similar to that used for the syn-

thesis of pteroylglutamic acid except that 2,3-dibromobutyraldehyde was used as a replacement for 2,3-dibromopropionaldehyde. The possibility of obtaining an analog of pteroylglutamic acid containing an additional methyl group was thus opened, indeed, Martin's group appeared to conclude that the product was "7-methylfolic acid." However, the reaction product made by Hultquist and Smith was evidently a mixture and has been designated "crude x-methyl pteroylglutamic acid." [3] The crude antagonist was found in experiments with various species to have profound biological effects which were completely reversible by pteroylglutamic acid. An experiment [4] which illustrates these effects is shown in Table 6.1. In this experiment, a purified diet was used containing 1 per cent succinylsulfathiazole and with pteroylglutamic acid omitted. Weanling female rats received this diet with various supplements and by using various levels of the antagonist and pteroylglutamic acid it was readily possible to show a competition between the antagonist in producing a specific syndrome and pteroylglutamic acid in preventing it. When the level of pteroylglutamic acid added to the diet was in excess of approximately 0.3 mg. per gram of antagonist, the rats were protected against pteroylglutamic acid deficiency. However, if this level was reduced, the animals developed signs of acute pteroylglutamic acid deficiency which became progressively more intense as the proportion of antagonist was increased and which were marked by slowing of growth, anemia, leucopenia and granulocytopenia. The almost complete disappearance of granulocytes in group 4 was particularly impressive. "Bloody whiskers" were observed in the deficient animals together with a severe diarrhea and necrotic and ulcerative changes in the oral cavity. The uteri were small and atrophic. Attention was drawn to the possibility of using the crude antagonist

to modify blood dyscrasias marked by erythrocytosis or leucocytosis. However, clinical experiments with a patient with chronic myeloid leukemia [5] indicated that the crude antagonist had very little biological potency for human subjects.

The synthesis of a far more potent antagonist, "4-amino pteroylglutamic acid" or "Aminopterin," was described in November, 1947 by Seeger, Smith and Hultquist.[6] In this compound the 4-hydroxyl group on the pteridine ring of pteroylglutamic acid is replaced by $-NH_2$ and this replacement was subsequently found to lead to a high degree of "anti-folic-acid" potency in a number of other analogs of pteroylglutamic acid in addition to Aminopterin. The

TABLE 6.1

Effects of Pteroylglutamic Acid (PGA) and Crude Antagonist on Hematology of Rats Fed Purified Diet Plus Succinylsulfathiazole (Franklin *et al.*)[4]

Group No.	*Supplement Per Kilo of Diet*		*Hemoglobin, Gm. Per 100 ml.*			*White Blood Cells Per cu. mm. x 10^{-3}*		*Granulocytes Per cu. mm.*		*Gain in Wt.*
	PGA Mg.	*Antagonist Gm.*	*1 wk.*	*2 wks.*	*3 wks.*	*3 wks.*	*5 wks.*	*3 wks.*	*5 wks.*	*4 wks.*
1	1	0	14.5	16.6	19.3	10.5	15.2	2400	2600	71
2	0	0	14.0	15.1	18.3	8.2	12.6	1500	1500	66
3	0	1	14.5	13.6	10.5	2.8	0.8	84	16	29
4	0	10	15.4	10.5	*	0.8	*	8	*	17
5	1	10	15.4	12.2	*	2.5	*	100	*	22
6	10	10	13.8	15.1	20.2	9.5	14.2	1200	1100	79
7	3	1	15.8	15.1	20.9	10.9	13.7	1500	1100	86
8	100	10	16.0	15.7	20.6	15.5	18.2	2900	1600	74

*Animals all dead.

latter compound was made by using 2,4,5,6-tetraminopyrimidine sulfate to replace 2,4,5-triamino-6-hydroxypyrimidine in the reaction by which pteroylglutamic acid is synthesized.[7] The substance was obtained in clusters of yellow

needles and in 0.1N sodium hydroxide solution it showed ultraviolet absorption maxima at 260, 284 and 370 mμ and minima at 239, 271 and 333 mμ. Aminopterin was soon shown to have the unprecedented property of producing a syndrome of vitamin deficiency which could not be reversed by the original metabolite, pteroylglutamic acid, although the antimetabolite differed from the metabolite only in having an amino group in exchange for an hydroxyl group. Death was produced in mice in about six days by feeding Aminopterin at a level of 1 part per million of diet.[8] The length of time of survival was reduced somewhat by raising the dietary level of Aminopterin. The effect was not reversed by feeding high levels of pteroylglutamic acid, although at a lower level of Aminopterin, 0.3 part per million of diet, some reversal of the toxicity was obtained. Studies with rats and chicks indicated a similar lack of reversibility.[9]

The use of Aminopterin in the treatment of acute leukemia in children was first described by Farber and coworkers.[10] Temporary remissions were produced in 10 of 16 cases. The white-cell count tended to return to normal level both in patients in whom the count was initially high and also in those who were leucopenic. The percentage of immature cells fell and the blast forms decreased markedly and in some cases disappeared from the bloodstream. The bone marrow showed changes varying from a disease in number of leukemic cells to their disappearance and from hypoplasia to an almost normal pattern. These changes in the direction of normality were only temporary and fatal relapses took place in a few months. Toxic effects, including stomatitis, were noted. Additional findings were described in a later publication,[11] and a number of other clinical investigators soon described similar results.[12 to 17] Diarrhea, alopecia, deafness and stomatitis were listed

among the toxic symptoms.[12] A summary of some of the published work is in Table 6.2. The temporary nature and short duration of any remissions produced by Aminopterin and its analogs should be emphasized in evaluating the treatment of leukemia.

TABLE 6.2

Remissions with 4-amino pteroylglutamic Acid (Aminopterin), 4-amino-10-methyl pteroylglutamic Acid (A-methopterin), and 4-aminopteroylaspartic Acid (Amino-an-fol), in Acute Leukemia, Principally in Children

No. of Cases	*No. of Remissions*	*Reference*
10	2	Jacobson, W., *et al.*[13]
8	5	Pierce, M., and Alt, H.[14]
54	8	Stickney, J. M., *et al.*[12,27]
	10%	Farber, S.[11]
35	9	Dameshek, W.[24]
8	3	Jimenez de Asaa, F.[25]
43	4	Meyer, L. M. *et al.*[26]
250	30%	Thiersch, J. B., and Philips, F. S.[28]
14	2	Sacks, M. S., *et al.*[29]
9	5	Smith, C. H., and Bell, W. R.[30]
13	9	Dacie, J. V., *et al.*[31]
27	6	Wilkinson, J. F., and Gardikas, C.[32]

A detailed study of the effects of injected Aminopterin on rats and mice was made by Philips and Thiersch.[18] The LD_{50} was 1.9 $\pm$ 0.3 mg. for mice and 4.5 $\pm$ 1.4 mg. for rats per kilo of body weight. Severe watery diarrhea appeared 48 hours after administering the drug in fatal doses with passage of blood in the terminal stages. Progressive weight losses were noted following the first day in rats receiving 40 mg. per kilo and changes in the femoral marrow of rats were noted by 12 hours, liquefaction becoming complete by 72 hours with disappearance of the hematopoietic tissues. Marked changes were noted in the intestinal tract, starting with venous hyperemia and followed by extensive desquamation of surface and crypt epithelium. Only moderate effects were produced on lymphoid tissues

in contrast to the action of nitrogen mustard. In another study with rats, Higgins [19] reported various pathological effects following the administration of Aminopterin including adrenal hyperplasia and atrophy of the thymus.

Other effects of Aminopterin include the following:

(1) Toxicity with characteristic lesions in dogs, monkeys and guinea pigs [20, 42] including diarrhea, peripheral leucopenia, depletion of bone marrow, abnormal nuclear disintegration of normoblasts, ileitis and ulcerative colitis in dogs.

(2) Inhibition of the growth of Rous sarcoma in chicks [21] and of sarcoma R-39 in rats.[22] Mice implanted with sarcoma 180 showed tumor inhibition and a reduction of normal erythropoiesis upon treatment with Aminopterin, but upon cessation of treatment both the erythropoietic process and the sarcoma 180 returned to their former state within 5 days.[23]

(3) Prolongation of the survival time of mice injected with transmitted leukemias AK4 and C1498.[33]

(4) Inhibition of estrogen-induced tissue growth in the female genital tract in frogs treated with estradiol,[34] in chicks treated with diethylstilbestrol, and in ovariectomized rats treated with estradiol.[35]

(5) Retardation of the rate of emergence and of survival in *Drosophila melanogaster*.[36] Toxic effects, including interference with mitosis, in onion seedlings *(Allium cepa)*.[37]

(6) Fetal death during early pregnancy in mice and rats. No significant signs of intoxication were observed in the mothers except temporary depletion of the bone marrow.[38]

(7) Reductions in the myeloid and red-cell series in the bone marrow of human patients together with dis-

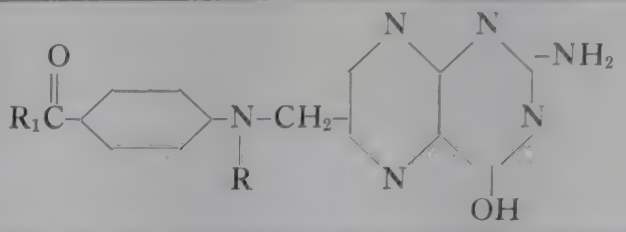
turbed mitosis and abnormal nuclear remnants. Six remissions of tumors of which five were lymphoid in origin were seen following periods of at least 10 days of treatment.[39] Of great interest in this investigation were observations on nine patients with tumors not involving the marrow. In all these cases, the total cellularity of the marrow decreased within 10 days of the onset of treatment and progressed to low levels with marked reductions in both the myeloid and red-cell series. In the latter, there was a decrease in the percentage of orthochromatic normoblasts from a normal of 80 per cent to less than 6 per cent. Increases were noted in macronormoblasts and in basophilic erythroblasts. Primitive erythroblasts and also megaloblasts appeared after prolonged treatment with the antifolic compounds; these cells developed into pathological hemoglobinized cells with giant red cells and irregular nuclear remnants. The changes observed should be considered in relation to the known role of pteroylglutamic acid in reversing megaloblastic arrest.

(8) Inhibition of progestational reactions in the uterus of mice, rats and rabbits.[40] When 4.5 micrograms of Aminopterin were injected daily into 20-gram mice for 28 days with 0.5 to 1.0 mg. of progesterone the characteristic effect of the hormone on the uterus was inhibited, but the inhibition was overcome by raising the dose of progesterone to 1.25 mg. daily. Similar inhibitions of the uterine response to progesterone were observed in rats and rabbits which received Aminopterin.

In a number of the above investigations, other 4-amino analogs of pteroylglutamic acid were used in addition to Aminopterin, including the 9-methyl, 10-methyl, 9,10-dimethyl, and aspartic acid analogs of Aminopterin. Other effects of Aminopterin are discussed in Chapters VII and VIII.

Table 6.3 (From [41])

N^{10}–substituted Pteroic and Pteroylglutamic Acids



R	R_1	*Purity*	*Antagonist Activity*[a]
$-CH_3$	$-OH$	Analytical	15.000
$-CH_3$	$-OH$	Crude	2.000
$-C_2H_5$	$-OH$	91.2%[b]	1.076
$-C_2H_5$	$-OH$	Crude	0.075
$-C_4H_9$	$-OH$	85%	0.135
$-C_4H_9$	$-OH$	Crude	...
$-CH_2COOH$	$-OH$	Crude	0.022
$-CH_2C_6H_5$	$-OH$	Crude	...
$-CH_2COC_6H_5$	$-OH$	Crude	0.010
$-CH_3$	−1 (+) glutamic acid	Analytical	100.000
		Crude	22.500
$-CH_2COC_6H_5$	−1 (+) glutamic acid	Crude	0.010

[a]An arbitrary value of 100 is assigned for the antagonist activity of N^{10}-methyl pteroylglutamic acid, for half-maximum inhibition of the growth of *Streptococcus faecalis* R. Values for other compounds are reported in terms of the standard.

[b]Estimated by ultraviolet absorption.

Table 6.4

Some Derivatives of Pteroylglutamic Acid and their Biological Effects

PGA Derivative	*Inhibition* of S. faecalis R at Three PGA Levels (in mug Per cc. of Culture Medium)*			*Toxicity for Animals, Expressed as ppm. of Purified PGA-deficient Diet for* LD_{100}			
	10	*100*	*1000*	*Mice*	*Rats*	*Chicks*	*Reversa*†
Crude "x-methyl"	30	20	30	1000	1000	1000	+
9-methyl	300	400	400	30 to 1000			+
10-methyl	1	1	0.8			Pro‡	
9, 10-methyl	3	2	2	100	30	30	
4-amino	6	3	2	1	1 to 3	3	−
4-amino-9-methyl	2	2	2		10		
4-amino-10-methyl	2	0.5	0.3	1	3	5	−
4-amino-9, 10-dimethyl	0.4	0.2	0.2	3	3	3	−

*Inhibition ratio to PGA for half-maximum growth.
†Reversible by PGA over a wide range.
‡Slight PGA-like effect.

TABLE 6.5

Classes of Pteroylglutamic Acid Analogs

Class Type	*Example*	*Reference*
1 Purine analogs	2-amino purine	43
2 Pteridine compounds	2, 4-diamino-6, 7-diphenyl pteridine	44 45
3 PGA with different substituents on pteridine nucleus or on side chain or on both	4-amino PGA	6
	N^{10}-methyl PGA	41
	4-amino-N^{10}-methyl PGA	46
4 Pteroylamino acids other than PGA	pteroylaspartic acid	47 48
5 PGA with modifications of pteridine nucleus	quinoxaline-2-carboxyl-yl p-aminobenzoyl-glutamic acid	49
6 PGA analogs with carboxyl of paba replaced by sulfonyl	N(-4-(((2-benzimidazol) methyl)-amino)-sulfonyl) glutamic acid	50

The synthesis of a series of pteroic and pteroylglutamic acid derivatives which were alkylated in the 10-position was described by Cosulich and Smith.[41] These compounds were made by using the appropriate p-alkylaminobenzoic acid in the synthesis [7] and they are listed in Table 6.3. Although 10-methylpteroylglutamic acid is a highly potent antagonist of pteroylglutamic acid for *S. faecalis* R (Table 6.4) it has a folic-acid-like effect for chicks on a deficient diet, perhaps due to oxidation or removal of the methyl group *in vivo*

Many analogs of pteroylglutamic acid can be synthesized by the appropriate chemical procedures, including the use of amino-acids other than glutamic acid, the alkylation of the amino group of the p-amino-benzoyl radicle, the use of the tetraminopyrimidine rather than the triamino hydroxypyrimidine and various other modifications. Some examples and references are listed in Table 6.5.

A more satisfactory understanding of the nature of the deficiency produced by Aminopterin came about as a result of the studies of the "citrovorum factor" (CF) which are discussed in Chapter VII. It appears that Aminopterin is a biological antagonist of CF as well as of pteroylglutamic acid. The profound changes produced by Aminopterin in living organisms constitute additional evidence for the importance of the folic acid group of compounds in biological systems.

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CHAPTER VII

THE CITROVORUM FACTOR

It was reported in 1948 by Sauberlich and Baumann [1] that certain natural materials, including liver extract and yeast, contained a growth factor for *Leuconostoc citrovorum* 8081 on a purified culture medium. The organism responded to thymidine, but it was concluded that some other active factor, the "citrovorum factor," was present in liver extract. A delayed and submaximum response was obtained with massive doses of pteroylglutamic acid. By comparative assays, "citrovorum factor" (CF) was differentiated from a factor required by the chick which is now known to be vitamin B_{12}. Lyman and Prescott [2] were able to separate CF and vitamin B_{12} in liver extract by observing that the two factors migrated in opposite directions in an electric field. The alkali-stability of CF [2] served to contrast it further with vitamin B_{12}. It was noted by Lees and Emery that *Le. citrovorum* would not respond to vitamin B_{12} and that CF was more heat-stable and alkali-stable than vitamin B_{12}.[3] A functional relationship between thymidine, pteroylglutamic acid and CF was suggested by Broquist and co-workers.[4] This relationship was emphasized by findings that natural materials containing CF would reverse the inhibitory effect of 4-amino pteroylglutamic acid (Aminopterin) for *Le. citrovorum* [5] and that thymidine but not pteroylglutamic acid would reverse the inhibitory effect

of Aminopterin for *Escherichia coli*.[6] It was shown by Sauberlich [7] that the administration of large doses of pteroylglutamic acid to rats or to a human subject increased as much as 200-fold the urinary excretion of CF. All of these findings suggested that CF was a biologically-active derivative of pteroylglutamic acid.

It was found by Bardos and co-workers [8] and by Broquist and co-workers [9] that CF, which was re-named "folinic acid" by the Texas group, was destroyed by very mild hydrolysis with hydrochloric acid and that a second compound with folic acid activity was simultaneously formed. "Folinic acid" was more effective than pteroylglutamic acid in reversing the inhibitory effects of "x-methyl folic acid" for *Streptococcus faecalis* R.[10] Concentrates of CF prepared from liver reversed the toxic effect of Aminopterin for mice [9] as shown in Table 7.1 and were able to replace pteroylglutamic acid in the nutrition of the chick.

TABLE 7.1

Effects of Pteroylglutamic Acid (PGA), Citrovorum Factor (CF) and 4-aminopteroylglutamic Acid (Aminopterin) on Growth and Survival of Mice on Purified Diet Containing No Added PGA

Group No.	*Supplements Injected Per Mouse Three Times Weekly*	*Weight and No. Surviving (In Parentheses)*		
		1 day	*5 days*	*9 days*
1	None	19.3(12)	20.2(12)	23.0(12)
3	10γ Aminopterin	19.5(12)	17.3(3)	(0)
4	20γ Aminopterin	20.0(12)	16.3(3)	(0)
6	10γ Aminopterin + 20γ PGA	18.0(12)	16.2(4)	(0)
7	20γ Aminopterin + 20γ PGA	19.2(12)	15.0(5)	(0)
9	10γ Aminopterin + 100,000 U CF*	19.2(12)	20.5(12)	19.5(12)
10	20γ Aminopterin + 100,000 U CF*	19.3(12)	18.0(8)	15.7(3)

*Equivalent to 20 micrograms of folic acid activity.

Lan and Sealock [11] found that liver slices from scorbutic guinea pigs did not exhibit the increased oxygen consumption in the presence of tyrosine which occurred in liver

slices from normal animals or when ascorbic acid was added *in vitro* to the liver slices from the scorbutic animals. A relation between pteroylglutamic acid and the oxidation of tyrosine was postulated by Rodney and co-workers [12] who found that small increases in the rate of oxygen uptake by homogenates of livers of pteroylglutamic acid-deficient rats were produced by adding tyrosine and that the increases were made somewhat greater when both pteroylglutamic acid and tyrosine were added. The influence of ascorbic acid and pteroylglutamic acid upon the disturbance of tyrosine metabolism which is produced in guinea pigs by scurvy was studied by Woodruff and Darby.[13] The high excretion of tyrosyl derivatives and of phenyl-pyruvic-acid-like compounds was decreased markedly by either ascorbic acid, 10 mg. daily, or pteroylglutamic acid which was effective at the rather high level of 5 to 15 mg. daily.

Nichol and Welch [14] found that ascorbic acid accelerated the formation of CF from pteroylglutamic acid by the liver slices of normal rats. About 2000 units of CF activity per gram of wet weight were found after incubation of the slices alone for two hours. This amount was doubled when 100 micrograms of pteroylglutamic acid or 10 mg. of ascorbic acid was added per gram, while a mixture of pteroylglutamic acid and ascorbic acid increased the level of CF three-to-five fold. The augmentations by pteroylglutamic acid and the mixture of pteroylglutamic acid and ascorbic acid were even more striking when liver slices from pteroylglutamic-acid-deficient rats were employed. The authors also stated that "the urinary excretion of CF, which is augmented by the administration of pteroylglutamic acid, is further increased from two-to-four-fold by doses of ascorbic acid ranging from 10 to 20 times that of pteroylglutamic acid."

In contrast to the effect of ascorbic acid, Aminopterin

was found to inhibit the formation of CF by rat liver slices [15] and Aminopterin also reduced the urinary excretion of CF in rats receiving supplementary pteroylglutamic acid. It was concluded that Aminopterin interfered with both the formation and utilization of CF.

A reaction mixture was prepared from pteroylglutamic acid by treatment with formic acid followed by reduction and was found to be active for *Le. citrovorum*.[16] A crystalline compound, "leucovorin," was prepared from pteroylglutamic acid by catalytic reduction over platinum in formic acid at 0° to 30°, followed by adsorption of impurities on Magnesol at pH 7, adsorption of activity on Darco G-60 at pH 4, elution, fractional crystallization of the barium salt and finally chromatography on Magnesol columns.[17] The barium salt of leucovorin had a composition corresponding to $C_{20}H_{21}N_7O_7Ba,5H_2O$ and an absorption spectrum in 0.1N sodium hydroxide solution with a maximum at 282 mμ and a minimum at 243 mμ. At pH 2 at room temperatures, leucovorin decomposed with loss of activity for *Le citrovorum* but retained folic acid activity for *S. faecalis* and *L. casei*. One CF unit corresponded to 0.15 millimicrogram of leucovorin expressed as the anhydrous free acid. The substance competitively reversed the lethal effects of Aminopterin in mice; about 30 micrograms of leucovorin completely prevented the toxicity of 10 micrograms of Aminopterin when injected simultaneously.

The finding that CF would reverse the toxic effects of Aminopterin aroused interest in the relation of CF to the anti-leukemic effects of Aminopterin and its close chemical relatives. It was found by Burchenal and co-workers [18] that the effect of 4-amino-N^{10}-methyl pteroylglutamic acid (Amethopterin) in prolonging the survival time of mice with transplanted leukemia AK4 was blocked almost completely by prior administration of leucovorin. About five

million units (0.75 mg.) of leucovorin reversed 2 mg. of the antagonist under these conditions but if the antagonist was injected one hour prior to the leucovorin the effectiveness of the latter was diminished.

Leucovorin was used by Schoenbach and co-workers [19] to combat the toxic effects of Aminopterin and Amethopterin in two patients who were receiving these folic acid antagonists for treatment of metastatic neoplasms. The first patient received Amethopterin, 10 mg. daily for the treatment of suspected metastases, following removal of a leiomyosarcoma. Ulcerated lesions, characteristic of Amethopterin toxicity, appeared in the mouth, and were accompanied by leucopenia. The administration of leucovorin, 6 mg. daily, was followed by healing of the ulcers in five days and by an increase in white blood cell count. The ulcers returned 10 days after cessation of the treatment with leucovorin. Similar results were noted with the second patient who was undergoing treatment with 2 mg. of Aminopterin daily.

Further studies relating to the excretion of CF were made by Broquist and co-workers.[20] On a normal diet the folic acid activity of urine corresponded to between 5 and 10 millimicrograms per ml. while the CF activity was equivalent to about 1 millimicrogram. It was suggested that an equilibrium between pteroylglutamic acid and CF might exist in the body. About 0.1 per cent of a 50 mg. dose of ingested pteroylglutamic acid was recovered in the form of urinary CF. The amount recovered was approximately tripled when 1 gram of ascorbic acid was given simultaneously with the pteroylglutamic acid. When 2.57 mg. of leucovorin was given orally, only 1.1 per cent of the dose was recovered as urinary CF and the recovery was lowered by the simultaneous administration of 1 gram of ascorbic acid. However, when leucovorin was given by

intravenous injection, 22 per cent of the dose appeared in the urine. The low recovery in the urine of the orally-administered material may have been due to destruction of leucovorin in the digestive tract, perhaps by gastric hydrochloric acid. The results are summarized in Table 7.2.

TABLE 7.2

Effect of Mode and Administration of Leucovorin on the Urinary Excretion of CF and Folic Acid Activity (FAA) in Adult Men

Subject	*Urinary Excretion in Six Hours following Oral Administration of 2.57 mg. Leucovorin Without Ascorbic Acid 1 gm. Ascorbic Acid*						*Urinary Excretion in Six Hours following Intravenous Injection of 3.05 mg. Leucovorin*		
	γ CF	*% CF Recovered*	*γ FAA*	*γ CF*	*% CF Recovered*	*γ FAA*	*γ CF*	*% CF Recovered*	*γ FAA*
1	28	1.1	50	4	0.16	18	682	22	630
2	39	1.5	63	13	0.5	29	679	22	970
3	32	1.2	66	12	0.5	20	725	24	620
4	31	1.2	51	19	0.7	30			
5	27	1.1	47	32	1.2	36	660	22	540
6	15	0.6	45				652	22	610
Average	29	1.1	54	16	0.6	27	680	22	674

EFFECTS OF CITROVORUM FACTOR (CF) ON THE MEGALOBLASTIC ANEMIAS

Emery and co-workers [21] stated that a concentrate of CF, free from vitamin B_{12}, gave no response in pernicious anemia but that leucocyte-stimulating activity was noted. The amount used was not stated.

The use of leucovorin in the treatment of five patients with pernicious anemia was described by Meyer and co-workers.[22] The first patient received 3 mg. of leucovorin intramuscularly daily and a submaximal reticulocyte response with slow increases in hemoglobin and red cell count were observed. The dosage was doubled and a secondary rise in

hemoglobin and erythrocytes was observed but the hematologic values did not reach normal levels after two months of treatment. The second patient also showed a submaximal reticulocytosis and slow increases in hemoglobin and erythrocyte values and there was some subjective improvement in neurologic status. The third patient had signs of neurologic involvement which disappeared upon treatment with leucovorin and hematologic improvement was observed. An excellent reticulocyte response occurred in the fourth patient together with marked increases in hemoglobin and red cell count, but death occurred in 47 days due to a coronary thrombosis. The fifth patient had an intercurrent infection and died following two transfusions and treatment with leucovorin, penicillin, streptomycin and large doses of vitamin B_{12}. The authors concluded that the administration of 1.5 or 3 mg. of leucovorin daily was followed by a submaximal reticulocyte response and the attainment of suboptimal levels of hemoglobin and red blood cells. The daily injection of 6 mg. produced a further rise in erythrocytes in three cases but normal blood values were not attained. Neurologic examinations were difficult to evaluate because of associated complicating diseases.

Responses were obtained to injections of leucovorin in four out of six cases of pernicious anemia by Ellison and co-workers [23] as shown in Table 7.3.

All three cases who received 0.75 mg. daily had maximal cell rises at three weeks despite submaximal reticulocyte peaks. Only case 5, who received 1.5 mg. daily had a maximal reticulocyte response. No changes in neurological status were noted in any of the patients during the three weeks of therapy. Four additional patients were given Aminopterin and either pteroylglutamic acid or vitamin B_{12} so that subsequent administration of leucovorin could be studied for its possible effect in reversing Aminopterin.

TABLE 7.3

Hematological Response in Pernicious Anemia to Leucovorin

Case Number	*1*	*2*	*3*	*4*	*5*	*6**
Leucovorin, mg. daily	0.75	0.75	0.75	1.50	1.50	1.50
Initial RBC, millions/cu. mm.	1.46	2.55	2.19	1.30	2.65	1.75
RBC at 3 weeks, millions/cu. mm.	3.09	3.56	3.27	2.51	3.75	
Expected RBC at 3 weeks, millions/cu. mm.	3.15	3.69	3.54	3.04	3.76	
Day of reticulocyte peak	10.00	10.00	11.00	8.00	7.00	None
Maximum reticulocytes %	12.70	5.40	6.60	6.90	9.80	
Expected max. reticulocytes %	24.50	10.00	16.00	27.00	9.00	20.50

*Subsequently received 120 micrograms vitamin B_{12} intramuscularly and responded with reticulocyte and RBC rise.

There was some indication that partial blocking of the effect of pteroylglutamic acid occurred, later reversed by leucovorin. Two patients were treated alternately with vitamin B_{12} and Aminopterin, one patient received 0.25 mg. of Aminopterin and 30 micrograms of vitamin B_{12} and 15 mg. of pteroylglutamic acid intramuscularly every other day for a 10 day period. The other patient received 1 mg. of Aminopterin and 10 micrograms of vitamin B_{12} on alternate days for a period of 10 days. Both patients had a maximal reticulocyte response. The latter patient is in contrast with a case described by Bethell,[24] who found that the simultaneous administration of 1 mg. of Aminopterin and 1 microgram of vitamin B_{12} daily for 16 days did not produce a response. The authors drew attention to the poor correlation between the reticulocyte responses and the erythrocyte responses in patients treated with leucovorin, suggesting that it may be possible that leucovorin lacks specific reticulogenic activity.

The response of two patients to leucovorin was described by Spies and co-workers.[25] One patient was described as having sprue. Both had initial red counts between 1.7 and 2.0 million. The patients received 3 mg. of leucovorin daily by intramuscular injection. Marked re-

missions occurred with reticulocyte peaks of 34 to 36 per cent on the eighth day.

The daily injection of 75 micrograms of leucovorin was found to produce marked responses in two cases of the megaloblastic anemia of infancy by Woodruff and co-workers.[26] The patients were 10-month-old fraternal twins. A marked increase in the number of normoblasts in the bone marrow was noted 72 hours after the beginning of therapy in the first infant, and a reticulocyte peak of 44 per cent was reached on the seventh day. Leucovorin therapy was continued for a total of 18 days. No secondary peak was produced later by another course of treatment with twice as much leucovorin. The second patient showed vitamin C serum levels which were so low as to approach those associated with clinical scurvy, but no scorbutic changes were seen in x-rays of the long bones. A rapid response was obtained to leucovorin with hematological changes similar to those in the first twin. The injections were discontinued after 13 days and no secondary response was produced to a subsequent injection of 5 mg. of pteroylglutamic acid. Two similar patients were treated by oral dosage with 500 and 200 micrograms of pteroylglutamic acid respectively and showed satisfactory responses. The minimum effective quantity of leucovorin administered parenterally was less than 75 micrograms; that of pteroylglutamic acid given orally was less than 200 micrograms so that it was not possible to decide which substance possessed the greater activity.

The treatment of three cases of pernicious anemia with leucovorin was described by Davidson and Girdwood.[27] The three patients received respectively, 3, 6 and 12 mg. of leucovorin in a single intramuscular injection and the hematological response was followed for two weeks. Only slight responses were noted in the first two cases but in the

patient who received 12 mg. a good response with increases in the hemoglobin from 6.5 to 9.8 gm. per 100 ml. and in the red count from 1.61 to 2.66 millions per ml. were observed in the 14-day period of observation; simultaneously there was a reticulocyte peak of 7.6 per cent on the fifth day and the sternal marrow was found to be normoblastic after seven days.

Three patients with pernicious anemia were treated by Jarrold and co-workers [39] with intramuscular injections of leucovorin for 10 days. Two of the subjects received 3 mg. daily and the other 1.5 mg. The authors concluded that "erythrocyte and hemoglobin rises were as good as would be expected with similar amounts of folic acid." The respective reticulocyte peaks were 9 per cent, 13.7 per cent and 23.1 per cent on the sixth and seventh days of treatment and the megaloblastic bone marrows were converted rapidly to a normoblastic pattern. One of the first two patients failed to respond to 0.6 mg. of leucovorin daily for 10 days before receiving the course of treatment with 3 mg. daily, leading to the conclusion that leucovorin was no more effective than pteroylglutamic acid.

CHEMISTRY AND CHEMICAL RELATIONSHIPS OF "CITROVORUM FACTOR"

The structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid was assigned to "folinic acid S.F." by Flynn and co-workers,[28] and the same formula was given to leucovorin by Cosulich *et al.*[29] (Figure 7.1). These findings reopened the question of the role of pteroylglutamic acid in the biological transfer of "single-carbon" fragments which had been the subject of speculation of Shive's group. These workers had noted that 4(5)-amino-5(4)imidazolcarboxamide (Figure 7.2) accumulated in the culture medium of

Escherichia coli when the growth of the organism was inhibited by sulfonamides. They concluded later that pteroylglutamic acid might be involved in the utilization of formate to produce hypoxanthine from the carboxamide as shown in Figure 7.2, basing the conclusion on (a) the isolation from natural sources [30] of a formylated substance in the folic acid group; "rhizopterin," 10-formylpteroic acid [31] and (b) the demonstration that 10-formylpteroylglutamic acid was more active than pteroylglutamic acid in reversing certain antagonists.[32] However, a further examination of the biological properties of 10-formylpteroylglutamic acid showed that, in contrast to leucovorin, it was as inactive as pteroylglutamic acid either as a growth factor for *Le. citrovorum* [1] or in reversing the toxic effects of Aminopterin for mice.[33] Speculation regarding the possible behavior of leucovorin in "transformylation"

$$\text{R-CHO} + \text{H}_2\text{O} \rightleftharpoons \text{R-H} + \text{HCOOH}$$

led to the conclusion that the equilibrium might lie between leucovorin and tetrahydropteroylglutamic acid rather than between pteroylglutamic acid and 10-formylpteroylglutamic acid. An examination of the biological properties of tetrahydropteroylglutamic acid lent support to this view. It is a white solid which may be prepared by catalytic hydrogenation of pteroylglutamic acid [34] and is readily oxidized by exposure to air, but it may be kept under nitrogen.

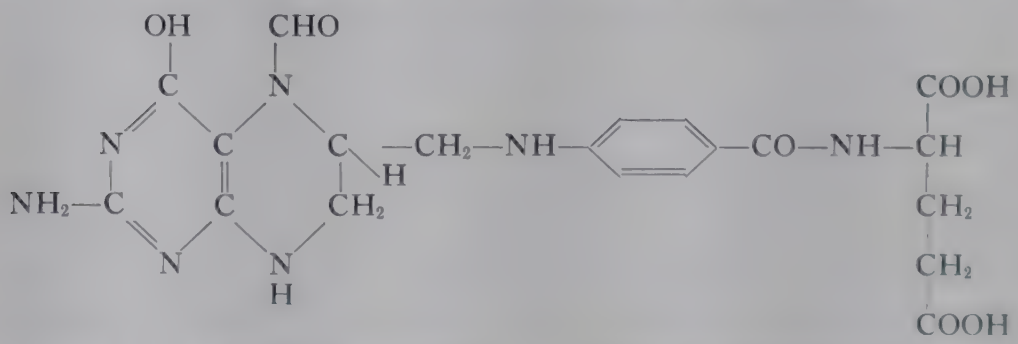


Figure 7.1. Leucovorin; 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.

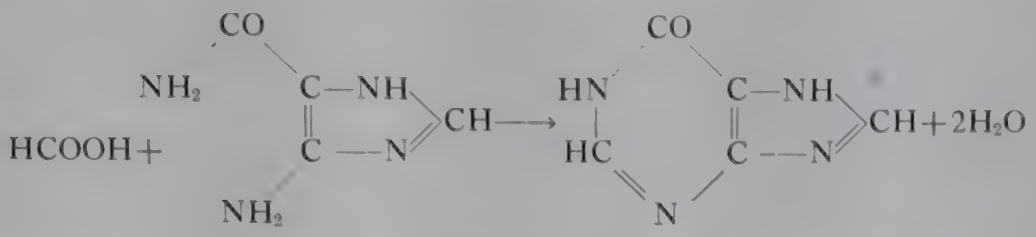


Figure 7.2. Formation of hypoxanthine from formic acid and 4(5)-amino-5(4)-imidazolcarboxamide.[35]

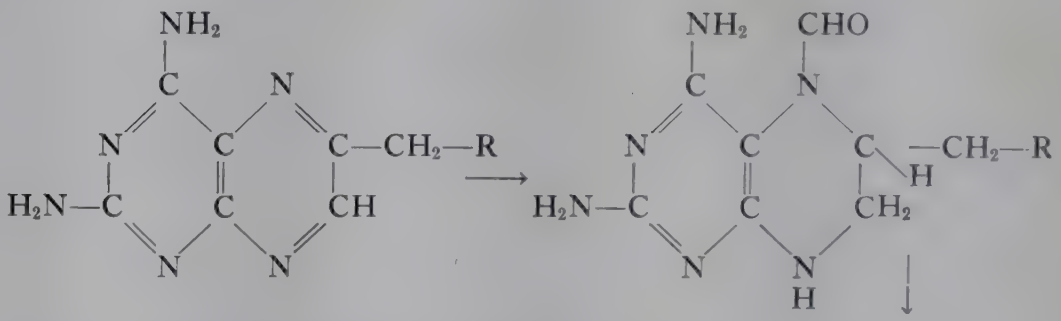
Tetrahydropteroylglutamic acid was found to promote the growth of *Le. citrovorum* when assayed by aseptic addition to the culture medium and to have an activity corresponding to about 2.5 per cent of that of leucovorin. Even more striking was the effect of tetrahydropteroylglutamic acid in protecting mice against the toxic effects of Aminopterin (Table 7.4). Tetrahydropteroylglutamic acid was about one-third as active as leucovorin in this protective action, and the effectiveness of these two compounds was in contrast to the inactivity of 10-formylpteroylglutamic acid.

TABLE 7.4

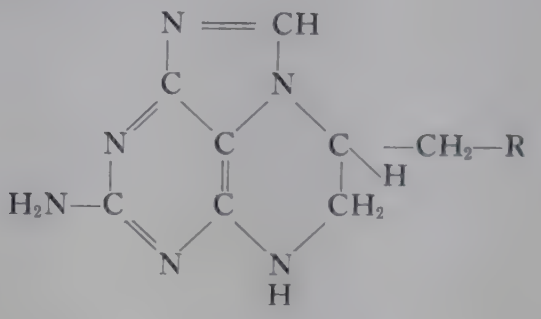
Growth and Survival of Male White Mice on a Purified Diet Deficient in Pteroylglutamic Acid as Modified by Various Supplements Injected Three Times Weekly

Supplement	*Wt. and No. Surviving (In Parentheses) (Days)*				*Average Survival Time (Days)*
	1	*6*	*7*	*8*	
None	18.7(12)	21.2(12)	21.6(12)	22.5(12)	
10γ Aminopterin	18.9(12)	14.2(16)	14.0(1)	all dead	4.9
10γ Aminopterin + 10γ leucovorin	19.1(12)	19.2(12)	19.0(12)	19.4(12)	
10γ Aminopterin + 20γ leucovorin	19.1(12)	21.3(12)	21.6(12)	22.6(12)	
10γ Aminopterin + 30γ tetrahydro PGA	18.5(10)	20.0(10)	19.4(10)	19.8(10)	
10γ Aminopterin + 100γ tetrahydro PGA	18.4(10)	21.5(10)	21.2(10)	21.7(10)	
10γ Aminopterin + 30γ 10-formyl PGA	18.2(12)	16.0(3)	14.0(1)	all dead	4.5
10γ Aminopterin + 100γ 10-formyl PGA	18.2(12)	14.3(3)	14.0(1)	all dead	5.7

Aminopterin has been shown to produce a diminution of the rate of utilization of formate in rats.[36] It seems conceivable that "blocking" of this biological reaction might result from the formation of an analogue of leucovorin from Aminopterin by reduction and formylation *in vivo*, followed by condensation of the 5-formyl group with the near-by 4-amino group to form an imidazole ring, thus giving rise to a compound which could displace leucovorin from the hypothetical enzyme system and which in contrast to leucovorin would be unable to transfer reversibly the "single-carbon fragment" represented by the 5-formyl group. These postulations may be formulated as follows:



Aminopterin: R=p-aminobenzoylglutamic acid



It was observed that an impure preparation of a dimethyl analogue of Aminopterin, 4-dimethylaminopteroylglutamic acid, promoted rather than inhibited the growth of *S. faecalis* R.[37] This compound in contrast to Aminopterin would be unable to form an imidazole ring by formylation of the 5-position and subsequent condensation.

Nichol and Welch [15] found that adding Aminopterin diminished the production of citrovorum factor activity from exogenous pteroylglutamic acid. This effect of Aminopterin was observed both in rat liver slices *in vitro* and in the urinary levels of citrovorum factor activity in rats which received various combinations of Aminopterin, pteroylglutamic acid and ascorbic acid. However, Aminopterin did not decrease the amount of citrovorum factor activity which was formed from tissue precursors. The authors commented that Aminopterin appeared to have much greater affinity than pteroylglutamic acid for the enzymes which produce citrovorum factor activity from pteroylglutamic acid.

Cosulich and co-workers [38] have isolated certain acid transformation products of leucovorin. The compounds are all converted into leucovorin by treatment with alkali and all can effectively replace leucovorin in the assay with *Le. citrovorum* when added aseptically. They reverse the toxic effects of Aminopterin when injected into mice.

A photomicrograph of crystals of the calcium salt of leucovorin is shown in Figure 7.3. Since in the synthesis of leucovorin a new asymmetric center is formed in position 6, it is probable that leucovorin is a racemic mixture with one-half the biological activity of "natural CF."

Some properties of concentrates of a preparation of citrovorum factor obtained from liver were different from those of leucovorin.[40] The liver preparation, calculated by a comparison of the absorption spectrum at 286 mμ in alkaline solution with that of leucovorin to be only 70 per cent pure, was about 15 per cent more potent microbiologically than leucovorin. Furthermore upon exposure to pH 2.0 for 20 hours at 23°, the liver preparation showed a loss of folic acid activity of 32 per cent for *S. faecalis* R while leucovorin showed an increase of 13 per cent. The obser-

vations may be explained by assuming that the "natural" citrovorum factor in liver is the biologically active enantiomorph with respect to position 6 while leucovorin contains 50 per cent by weight of the unnatural and biologically inert enantiomorph. Treatment of "natural" citrovorum factor with dilute acid in the presence of air would tend to change the factor to 10-formyl PGA by migration of the CHO group from position 6 to position 10,[38, 41] followed by dehydrogenation of the tetrahydropyrazine ring.[41] Since 10-CHO PGA is less potent than PGA (table 3.1) which in turn may be approximately as active by weight as "natural" CF,[40] a diminution in biological activity would be encountered. Treatment of leucovorin with dilute acid would bring about the same changes, but these changes would lead to an *increase* in the potency of the unnatural enantiomorph which by transformation of the optically active center at position 6 into an inactive center by de-

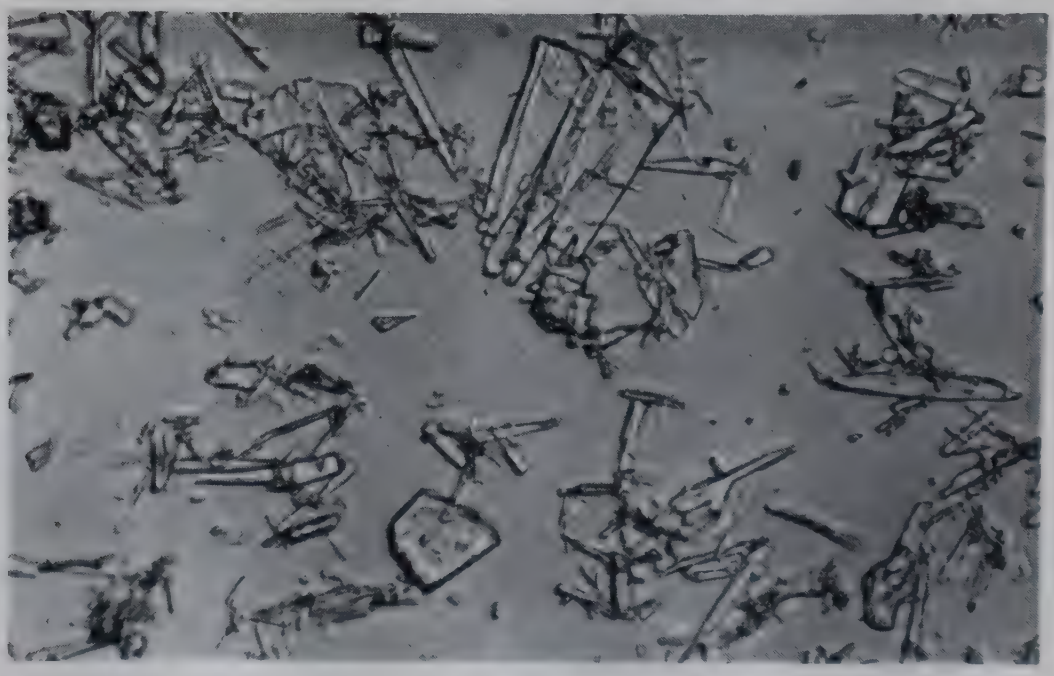


Figure 7.3. Photomicrograph of the crystalline calcium salt of leucovorin; calcium DL-5-formyl-5,6,7,8-tetrahydropteroyl glutamate ×800. (Calco Chemical Division, American Cyanamid Co.)

hydrogenation would be converted to biologically-active 10-formyl PGA, so that the over-all activity of leucovorin for *S. faecalis* R would be enhanced by treatment with dilute acid in the presence of oxygen.

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CHAPTER VIII

METABOLIC REACTIONS INVOLVING FOLIC ACID AND VITAMIN B_{12}

It was found that a relation existed between thymine (5-methyl uracil) and "folic acid" in the nutrition of *S. faecalis* R.[1] The organism was able to grow when thymine and a purine base were added to the culture medium or when a "folic acid" preparation replaced this combination. Uracil could not replace thymine in the absence of folic acid. Studies with *L. casei* [2] showed that growth could take place in the presence of thymine plus either guanine, adenine, hypoxanthine or xanthine. Uracil or cytosine could not replace thymine, but the thymine-purine mixture was unnecessary if a factor, later shown to be pteroylglutamic acid,[3] was added instead. These and subsequent observations [4] led to the conclusion that the function of pteroylglutamic acid in the nutrition of certain lactic acid bacteria was to enable the organisms to synthesize thymine.

The synthesis of thymine in adult rats was found by Elwyn and Sprinson [5] to proceed by methylation of the 5-carbon atom of a pyrimidine nucleus. The carbon atom could originate from α-C^{14}-labeled glycine or from β-C^{14}-labeled serine. These compounds are known to give rise to labeled formate and pteroylglutamic acid is concerned in these transformations.[6] It can therefore be postulated that a pteroylglutamic-acid-like compound, perhaps leucovorin,

(Chapter VII) is concerned with the transfer of a "single-carbon fragment" in the methylation of the pyrimidine ring to form thymine.

It appears that a similar relationship exists in the formation of thymidine. This substance, but not the other purine or pyrimidine desoxyribosides, will replace "citrovorum factor" for the growth of *Le. citrovorum*.[7, 8] Thymine is ineffective. The inhibitory effect of Aminopterin on the growth of *E. coli*[9] or of *Le. citrovorum*[10, 11] is reversed non-competitively by thymidine, thus indicating that thymidine is the end-product of a biological reaction which is blocked by Aminopterin in these bacterial species. Thymidine can serve as a precursor of thymine in rats.[12]

The involvement of pteroylglutamic acid in the addition of formate to aminoimidazolcarboxamide in the biological production of hypoxanthine was first observed by Shive's group and was discussed in Chapter VII. Woolley and Pringle[13] found that the carboxamide accumulated in the culture medium when *E. coli* was grown in the presence of amounts of Aminopterin which were sufficient just to inhibit multiplication slightly. Thus either sulfonamides or Aminopterin appeared to block the synthesis of purines at the same point, which led the authors to suggest that the sulfonamides act by inhibiting the formation of pteroylglutamic acid and consequently the production of the purines. This finding was confirmed by Edwards and co-workers who also noted that no evidence, as based on colorimetric tests, for the formation of the carboxamide was found when other inhibitors including 2,6-diaminopurine, 8-azaguanine, urethane and a nitrogen mustard were used in the culture medium, indicating a specific role for the pteroylglutamic acid mechanism in the formation of purines.[14] Recently Schulman and Buchanan[15] have used C^{14}-formate and the carboxamide to follow the formation of inosinic acid by

pigeon liver preparations. They found that the incorporation of the C^{14}-atom into inosinate was accelerated by pteroylglutamic acid and especially by leucovorin. Their results suggested the existence of an equilibrium reaction in which inosinate cleaved to formate and carboxamide ribotide which then reacted with tagged formate yielding inosinate labeled in the 2-position.

The administration of pteroylglutamic acid antagonists has been found to inhibit the synthesis of nucleic acids and to decrease the utilization of formate in the synthesis of nucleic acids.[16, 17] Incorporation of $C^{14}O_2$ into purines was partially blocked, but no decrease in the non-specific fixation of CO_2 by the tissues was observed.[17]

A relation between pteroylglutamic acid and the formation of serine was indicated in studies with certain bacteria [18, 19] and with rats.[6] At about the same time, a number of investigators showed by means of isotopes that an interrelationship exists *in vivo* between glycine, formate and serine as follows [20, 21, 22, 23, 24]

$$\overset{*}{C}H_2NH_2COOH \longrightarrow H\overset{*}{C}OOH + \overset{*}{C}H_2NH_2COOH \rightleftharpoons \overset{*}{C}H_2OH\overset{*}{C}HNH_2COOH$$

A deficiency of pteroylglutamic acid slows the rate of these transformations. The deficiency has been reported to lead to diminutions in the rates of various biological reactions including the conversion of serine to glycine in rats,[25] the incorporation of radioactive glycine in chick liver homogenates [26] and the incorporation of formate-carbon into serine and other amino-acids in rats.[6] In contrast, biotin deficiency did not influence the incorporation of C^{14} from formate into the β-carbon of serine in rats.[6]

The addition of both pteroylglutamic acid and vitamin B_{12} is necessary to enable rats to grow on a "labile-methyl-free" diet containing homocystine. The need for two un-

identified factors first became evident in studies by Bennett and Toennies [27] who found that a crude liver extract permitted the growth of rats on such a diet while no growth was obtained when a more refined and concentrated liver extract was used. The latter liver extract, which contains about 20 micrograms of vitamin B_{12} per ml., was shown to be almost completely free from folic acid activity.[28] Later it was shown by Bennett that both vitamin B_{12} and pteroylglutamic acid were needed to produce growth in rats at a rate of 0.8 to 1.0 gram daily on a "labile-methyl-free" diet containing homocystine and succinylsulfathiazole. Since the diet contained glycine and serine, it would appear that these substances might serve as sources of formate for the methylation of homocystine to form methionine [24, 29] and that vitamin B_{12} and pteroylglutamic acid might be needed for the catalysis of the reactions involved.

A function for vitamin B_{12} in the formation of methionine from homocystine was shown by Shive who found that the inhibitory effect of sulfanilamide on the growth of *E. coli* was overcome by the addition to the culture medium of either vitamin B_{12} at a level of 0.3 microgram per liter or by methionine at a level of about 90 mg. in contrast to homocysteine which did not affect the inhibition index. A catalytic role for vitamin B_{12} in the formation of methionine from homocysteine was indicated from these results which were substantiated by the work of Davis and Mingioli [30] who studied a number of mutants of *E. coli* which required either vitamin B_{12} or methionine for growth but which would not respond to homocysteine. By growing the mutants on a medium containing vitamin B_{12} it was shown that a substance was produced which promoted the growth of other mutants which were known to respond to homocysteine. These results also indicated that vitamin B_{12} was involved in the conversion of homocysteine to methio-

nine and that the former substance accumulated in the mutant which was unable to effect the conversion. It was noted by Jukes and co-workers [31] that vitamin B_{12} appeared to be concerned in the formation of methionine from homocystine by chicks. Homocystine, with or without betaine, did not promote the growth of vitamin-B_{12}-deficient chicks on a purified diet which was deficient in methionine. The chicks responded to the addition of methionine. However, when the chicks received vitamin B_{12} they showed a growth response to either methionine, homocystine or homocystine plus betaine.

Both vitamin B_{12} and folic acid have been implicated in the response of the chick to choline. Schaefer and co-workers [32, 33, 34] studied the growth of chicks on a diet which was deficient in both vitamin B_{12} and methionine and which contained added pteroylglutamic acid. They found that growth was more rapid when the level of choline was 0.6 per cent than when the level was 0.1 per cent. However, when a source of vitamin B_{12} was added, growth was as rapid with 0.1 per cent choline as with higher levels. It was subsequently noted that conversely when vitamin B_{12} was included in the basal diet and folic acid was omitted, the level of choline required for rapid growth was greater in the absence of added pteroylglutamic acid than in its presence.

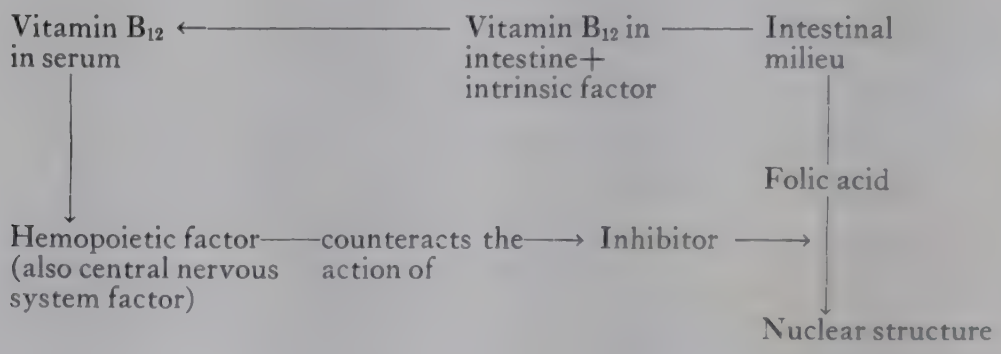
Gillis and Norris [35] compared the effects of various supplements on the growth of chicks on a basal diet of vegetable origin. A growth response was obtained with either 2 grams of betaine or 2 grams of choline per kilogram of diet or with 1.5 grams of a liver preparation containing about 1.7 micrograms of vitamin B_{12} activity per gram. No additional growth response to betaine or choline was obtained when the liver preparation was included in the diet and similar results were obtained when vitamin

B_{12} was used instead of the liver preparation.[36] It was noted by Jukes and Stokstad [37] that the requirement for choline for maximum growth of chicks was greater in the absence of vitamin B_{12} than its presence, but the amount of choline required for the prevention of leg deformities was not decreased by supplying vitamin B_{12}.

Deficiencies of either pteroylglutamic acid or vitamin B_{12} were found to slow the rate of utilization of C^{14} formate or α-C^{14} glycine for the synthesis of choline and cysteine.[38] The rate of uptake of C^{14} into the methyl group of choline was smaller than the uptake into the ethanolamine side chain, particularly in vitamin B_{12} deficiency. Homogenates of livers of rats deficient in vitamin B_{12} or in vitamin B_{12} and pteroylglutamic acid had a much lower capacity than normal for the formation of methionine from homocysteine and either choline or betaine, while liver homogenates from rats deficient in either pteroylglutamic acid or vitamin B_{12} and pteroylglutamic acid showed a reduced ability to form cysteine from homocysteine and serine although the cleavage of cystathionine to cysteine was not affected. The addition of vitamin B_{12}, pteroylglutamic acid or leucovorin to the deficient homogenates was without effect.

Certain relationships between pteroylglutamic acid, leucovorin and the oxidation of tyrosine were discussed in Chapter VII. A disturbance in tyrosine metabolism in premature infants is marked by the excretion of large amounts of hydroxyphenyl derivatives in the urine when high-protein diets are fed.[39] The administration of ascorbic acid decreases the rate of excretion of these derivatives. It was found by Govan and Gordon [40] that administration of 5 mg. of pteroylglutamic acid decreased the excretion of "tyrosyl" compounds in four out of 10 premature infants. Ascorbic acid was effective in producing decreases in five of the remaining six infants.

The relation of various factors to the conversion of human megaloblasts to normoblasts in bone marrow cultures *in vitro*, has been studied by Lajtha and others.[41, 42, 43, 44] The serum of patients with pernicious anemia in relapse was found to contain a factor which inhibited this conversion and the action of which diminished upon dilution.[41, 42] In contrast, normal serum contained a factor which increased the rate of ripening of the megaloblasts. Furthermore, megaloblasts were developed in normal bone marrow cultures which were incubated with pernicious anemia serum. The inhibitory effect was not destroyed by heating pernicious anemia serum to 56° for one to two hours before culturing the marrow preparations. Pteroylglutamic acid, 2 micrograms per ml. of medium, was found to have a ripening effect on the cultures in both normal and pernicious anemia sera, but vitamin B_{12} was completely ineffective. The following scheme was postulated to account for the experimental observations:



According to this, the lack of intrinsic factor in pernicious anemia results in a defect in the absorption of vitamin B_{12} from the intestine which leads to a deficiency of the hypothetical "hemopoietic factor." This deficiency results in a failure to counteract the inhibitory factor so that the action of folic acid is inhibited. The administration of folic acid overcomes this inhibition by mass action, and the

injection of vitamin B_{12} leads to the formation in the tissues of the "hemopoietic factor" which overcomes the action of the inhibitor and in addition arrests the central nervous changes.

Further studies [44] indicated that leucovorin had a direct ripening effect on megaloblasts *in vitro* at concentrations of 0.1 and 0.01 microgram per ml. of medium. Since pteroylglutamic acid was not tested at such low concentrations, no comparison of the activities of leucovorin and pteroylglutamic acid was possible.

These results are in contrast with *in vivo* responses which were reported by Horrigan and co-workers [45] who instilled solutions of various substances into the marrow cavities of the iliac crests of six patients with pernicious anemia in relapse. Bone marrow was aspirated from the marrow cavities immediately before and 48 hours after the instillation of the test substance. It was found that a decrease in the number of megaloblasts and an increase in normoblasts occurred close to the site of injection following the instillation of 1 microgram of vitamin B_{12} but pteroylglutamic acid, 1 or 2 mg., was ineffective. No such change took place in marrow obtained from the contralateral iliac crest following the instillation of 1 microgram of vitamin B_{12}, however the instillation of 15 micrograms was followed by a response in the marrow of the opposite ilium. In further studies,[46] leucovorin was found to be ineffective in producing a "local" response when respective amounts of 0.006 mg., 1.5 mg. and 3 mg. were instilled in three patients.

A relation between pteroylglutamic acid and the metabolism of estrogens was first shown in experiments with chicks. A dietary deficiency of pteroylglutamic acid in female chicks led to a failure in the anticipated response of the oviduct to diethylstilbestrol.[47] The chicks were placed

on a purified diet which was deficient in pteroylglutamic acid, and, in one group, pantothenic acid was also omitted from the diet. Some of the results are shown in Table 8.1. The estrogen-treated chicks received 0.5 mg. of diethylstilbestrol in 0.1 ml. of corn oil subcutaneously on each of the six days preceding autopsy. A good response to diethylstilbestrol was observed in pantothenic-acid-deficient chicks, thus indicating a specific connection between pteroylglutamic-acid-deficiency and the failure to respond to the estrogen. It was found [48] that sexually immature rhesus monkeys failed to respond to dosage with estradiol benzoate when maintained on a purified diet which was deficient in pteroylglutamic acid.

The administration of antagonists of pteroylglutamic acid was found to reduce the response to estrogens in the chick [49, 50] and the frog.[51] A diminished response to testosterone in the seminal vesicles and coagulating glands of mice receiving an antagonist, "x-methyl" pteroylglutamic acid, was reported by Goldsmith and co-workers.[52] In contrast, the effect of testosterone in stimulating comb growth in chicks was not abolished by adding the same antagonist to the diet.[53]

Increasing the protein level of the diet was shown by Cary and Hartman [54] to accentuate a deficiency syndrome in rats which was later shown to be due to a lack of vitamin B_{12}. The deficiency was found to be associated with elevated concentrations of blood non-protein-nitrogen in chicks [55] and rats.[56] A uremia occurring in rats newly born of mothers on a vitamin-B_{12}-deficient diet was prevented by injecting the young subcutaneously with 0.05γ of vitamin B_{12} shortly after birth.[57]

A role for vitamin B_{12} in reducing the S-S group in homocystine during its conversion to methionine was suggested by Dubnoff [58] who found that the addition of vita-

min B_{12} to liver slices favored the reduction of glutathione and homocystine and postulated that vitamin B_{12} maintained homocysteine in the reduced state, thus favoring its methylation. Oginsky [39] was unable to show an effect of vitamin B_{12} in a liver brei-homocystine-betaine system *in vitro*.

TABLE 8.1

Oviduct Weights in Chicks at 21 to 25 Days as Related to Diethylstilbestrol and to Deficiencies of Pteroylglutamic Acid and Pantothenic Acid[47]

Supplemental Treatment	*Body Weight, Gm.*		*Oviduct Weight, Mg.*	
	Range	*Mean*	*Range*	*Mean*
No pteroyltriglutamic acid*	53 to 90	71	32 to 82	62
20 mcg. pteroyltriglutamic acid daily from hatching	111 to 166	140	309 to 777	450
20 mcg. pteroyltriglutamic acid daily for last 10 days	59 to 158	110	84 to 474	196
No pantothenic acid; 20 mcg. pteroyltriglutamic acid daily from hatching	60 to 80	70	156 to 553	281
No diethylstilbestrol injected	97 to 144	117	19 to 33	25

*Pteroyltriglutamic acid (PTGA) was used as a source of pteroylglutamic acid.

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CHAPTER IX

VITAMIN B_6 DEFICIENCY AND ANEMIA

An anemia which developed in young dogs on a purified diet was described by Fouts and co-workers.[1] The blood dyscrasia occurred in the absence of a source of the "rat antidermatitis factor" which was later identified as pyridoxine. The dogs developed a severe microcytic hypochromic anemia; the hemoglobin was 12.6 to 15.3 grams per 100 cc. of blood at the start of the experiment and decreased to 2.6 to 3.6 grams. Simultaneously, the red blood cell count fell to about 36 per cent of its original value and the mean erythrocyte diameter decreased by about 25 per cent. The leucocyte count remained normal. One dog had three convulsions while anemic, and hyperplastic bone marrow was noted in one animal. The addition of rice polish extract containing the factor to the diet of three dogs was followed by a rise in reticulocytes and the rapid disappear- of the microcytic hypochromic anemia. Marked reticulocyte responses occurred which reached peaks on the second or third day of therapy. Subsequently[2] the anemia was shown to respond in a similar manner to crystalline pyridoxine. The mean corpuscular volume was 71 to 74 cubic microns at the beginning of the experiment and dropped to 48 to 54 cubic microns during the deficiency. Following therapy the value rose to 62 to 67 cubic microns. Elevated plasma iron levels were noted in a further study.[3]

Analogous observations were made with pigs [4, 5, 6, 7, 8] and were discussed by Cartwright in his comprehensive review.[9] The anemia was severe; the hemoglobin falling as low as 1.4 grams per 100 cc.; anisocytosis was marked, large polychromatic red corpuscles appeared but were outnumbered by the microcytes. The bone marrow was hyperplastic and became normoblastic following therapy. Ataxia and convulsions occurred and degeneration was found in the peripheral nerves, the spinal ganglia, the posterior roots, and the dorsal funiculi of the spinal cord. Hemosiderosis of the spleen, liver and bone marrow was found and could be prevented by restricting the intake of iron although the anemia was aggravated by this procedure. As with dogs, marked and rapid responses were produced in swine by the administration of pyridoxine hydrochloride. Intravenous dosage produced the greatest responses but the daily feeding of amounts as low as 80 micrograms per kilo of body weight was followed by a definite and pronounced remission. About one-third of the members of a group of pyridoxine-deficient rats were found to have anemia by Kornberg and co-workers [10] and all rats showed an abnormally slow rate of blood regeneration following bleeding. Pyridoxine is concerned in the metabolism of tryptophane [11] and its biologically related analogues pyridoxal and pyridoxamine function in the decarboxylation of amino acids [12] and in the transaminase system.[13] In spite of the profound nature of pyridoxine deficiency in experimental animals and of the important role of the vitamin B_6 group in biochemical processes, a definite syndrome due to a deficiency of this vitamin has not been recorded in human beings. Disturbances in tryptophane metabolism were observed in two human subjects after two to three weeks on a diet deficient in vitamin B_6.[14] An increase in xanthurenic acid excretion following dosage with tryptophane was noted; the increase

was eliminated after treatment with pyridoxine. McGarrity and co-workers [15] studied the effect of pyridoxine on hyperemesis gravidarum and found that their patients showed a lowering of the blood urea level which was restored to normal by pyridoxine. Changes in blood urea after a test load of alanine differed from normal and the changes were brought back to a normal pattern by administering pyridoxine.

Pyridoxine deficiency in two mentally defective infants was described by Snyderman and co-workers.[16] The infants were kept on a purified deficient diet, pyridoxic acid disappeared from the urine, the infants lost the ability to convert tryptophane to nicotonic acid and one subject developed a hypochromic anemia at approximately the 130th day. This responded remarkably to pyridoxine; a rise in reticulocytes was noted after 72 hours reaching a peak in 4 days, after which the red cell count and hemoglobin rose to normal. The other subject developed a series of convulsions on the 76th day which necessitated treatment with pyridoxine so that anemia was not observed in this second case.

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INDEX

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X

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B-VITAMINS
For Blood Formation

By THOMAS H. JUKES, PH.D.

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